Phylogeny, phylogeography and movement of *Kirramyces* spp. associated with leaf blight diseases of plantation eucalypts

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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May 2008

Declaration

I declare that this thesis is my own account of my research and contains as its main content work, which has not been submitted for a degree at any tertiary education institution.

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Acknowledgements

I would particularly like to thank my supervisors, Dr Treena Burgess and Assoc. Prof. Giles Hardy for their enthusiasm, guidance and support during my study. I am also very grateful to Prof. Mike Wingfield for his help and advice, generosity in funding and supporting my research visits to South Africa.

My special thanks go to Dr Paul Barber for his help and advice with morphological aspects of the study. Many thanks to Dr Angus Carnegie for collecting the leaf material from New South Wales for me and his help and advice. Thanks to Geoff Pegg for collecting the leaf material from Queensland for me and collaboration on papers. Thanks to Prof. Bernie Dell for his input on *Kirramyces destructans* in Asia and General discussion. Many thanks to Alex George for editing the Latin description of new species. Thanks to Margaret Dick for providing isolates of *Kirramyces eucalypti* from New Zealand.

I am grateful for the Murdoch University scholarship that I received throughout my studies. Also thanks to ARC Discovery grant no DP0664334 for operating costs.

Special thanks to Gavin Hunter, Maria-Noel Cortinas and Irene Barnes for their help and advice during my visits to FABI, South Africa. I wish to thank culture collection technician; Martie Van Zyl from FABI, South Africa for her help with cultures. Thanks to my friends from FABI, South Africa; Draginja Pavlic, Marija Kvas, Derian Echeverri, Ryan Nadel for their support and entertainment during my visits to South Africa.

Many thanks to my lab and coffee mates; Dianne White for technical assistance, Monique Sakalidis and Kate Talyor.

Thanks to the people who lent me herbarium specimens thus, made this work possible; Dr Michael Priest (DAR), Ms. Milena Ross (IMI), Dr Eric McKenzie (PDD).

There are many people that I want to thank for that were not directly included in this work but helped me to get to this stage; my never forgotten ex-boss and friend Dr Sandra Jackson who unfortunately will not see this thesis, my masters thesis supervisor Dr Anthony Cole (University of Canterbury, New Zealand), Dr John Klena (US Naval Medical Research, Cairo, Egypt), Prof Krishnapillai Sivasithamparam (University of Western Australia) who initiated my move to Perth.

Finally, last but not least thanks to my husband Zarko my son Vuk and my parents for their support during the long hours I have put into my studies.

ABSTRACT

When this study commenced in early 2004, only five *Phaeophleospora* species. had been reported from eucalypts of which only two; *P. destructans* (STE-U 1336) and *P. epicoccoides* (STE-U 1346) had been sequenced. In a former study, *Phaeophleospora* species emerged in two separate clades suggesting that *Phaeophelospora* is polyphyletic. The appearance and severity of lesions on eucalypt leaves are generally used to recognise the species of *Phaeophleospora* responsible for disease. However, depending on host and climate, the symptoms associated with infection by *P. epicoccoides*, *P. eucalypti* and *P. destructans* can be almost identical and incorrect diagnosis is a common problem. Thus, *Phaeophleospora* species were compared based on DNA sequences and multi gene genealogies were constructed. In addition species-specific primers were designed and tested on leaf material.

Many isolates of *Phaeophleospora* spp. were collected and sequenced, and all *Phaeophleospora* spp. from eucalypts were shown to cluster together and are closely related to the most important leaf pathogens associated with eucalypts namely *Colletogloeopsis zuluensis*, *Mycosphaerella cryptica* and *M. nubilosa.* In contrast, these fungi are distantly related to the type specimen of the genus *Phaeophleospora*, *P. eugeniae*. Furthermore, all DNA sequences of isolates of *P. destructans* examined in this thesis, including the ex-type culture, were identical but different to one previously lodged in GenBank.

This phylogenetic separation led to a morphological study of the species assigned to *Phaeophleospora* and compared the species from eucalypts with *P. eugeniae* the type specimen of *Phaeophleospora*. The phylogenetic and morphological studies show that *P. eugeniae* is well separated from *Phaeophleospora* spp. occurring on eucalypts and led to the resurrection of the previous generic name, *Kirramyces* for *Phaeophleospora* spp. occurring on eucalypts. Furthermore, phylogenetic analysis and morphological observation of *Kirramyces* spp. and *Colletogloeopsis* spp. occurring on eucalypts showed considerable overlap between these two genera. Therefore, *Colletogloeopsis* was reduced to synonymy with *Kirramyces*. Consequently, the genus *Kirramyces* was expanded from five to 14 species, and included the description of two new species, *K. angophorae* and *K. corymbiae*. In order to

assist with their identification a key based on morphology of conidia for *Kirramyces* species was developed.

Kirramyces destructans is a devastating pathogen originally described from Indonesia in 1996 and has since been found throughout Asia where all common tropical and subtropical plantation eucalypt species and hybrids are susceptible. *K. destructans* is considered a major biosecurity threat in Australia, both to native eucalypt forests and the tropical plantation industry. Prior to the current study, there had been no investigation into the origin and movement of this important pathogen. Thus, five gene regions and six microsatellite loci were sequenced for 43 representative isolates of *K. destructans* from a range of geographical locations and hosts. Two microsatellite markers detected very low nucleotide polymorphism (three haplotypes for each loci); five other gene regions, including four microsatellite region were uniform. This low level of genetic diversity provides strong evidence that *K. destructans* was introduced into Indonesia as a founder population and that it has subsequently been spread throughout Asia via human-mediated movement of germplasm. Timor and Northern Australia were considered to be a possible source of origin of this fungus, but the high susceptibility of native *E. urophylla* to *K. destructans* in Timor indicates that the pathogen is unlikely to be endemic to Timor.

The current distribution of *Kirramyces eucalypti* is New South Wales, Queensland, Victoria, Tasmania and New Zealand (North Island). The main host of this pathogen is *E. nitens* which is native to Victoria and New South Wales. *Kirramyces eucalypti* has not been found in South Africa, yet it causes a severe disease on eucalypt hybrids originating from South Africa growing in New South Wales indicating movement to these hybrids from either native eucalypts or nearby plantations. As such, *K. eucalypti* poses a threat for the plantation industry in sub-tropical and tropical Australia. The phylogeography of *K. eucalypti* in Australia and New Zealand was studied by sequencing three gene regions and one microsatellite locus of fifty-seven representative isolates of *K. eucalypti* from Queensland, New South Wales, Victoria, Tasmania and New Zealand. The highest genetic variation was found among isolates from NSW suggesting that *K. eucalypti* originates from NSW. Isolates in New Zealand appear to have been introduced from NSW. Isolates from Queensland were consistently different to those from other regions and may in fact represent a cryptic species or a hybrid.

During monitoring of eucalypt taxa trials in far North Queensland, infected leaves resembling symptoms typical of *K. destructans* were collected and examined. Phylogenetic data based on three gene regions and some morphological characteristics revealed a new taxon described in this study as *K. viscidus. Kirramyces viscidus* was also shown to be closely related to the devastating pathogen *K. destructans*. *Kirramyces viscidus* had been found to cause extensive damage to eucalypt hybrids originating from South America, and less damage to *E. grandis* from Australia, indicating that this pathogen is probably endemic to Australia. *Kirramyces viscidus* has the potential to seriously damage tropical eucalypt plantations, especially if clonal and planted off-site.

In conclusion, this study resurrected genus *Kirramyces* for the *Phaeophleospora* and *Coletoglloeopsis* spp. occurring on eucalypts. It also studied the phylogeography and gene flow of the two most important *Kirramyces* species, *K. destructans* and *K. eucalypti* and describes three new *Kirramcyes* spp. found on eucalypts in Australia. Very recently, *K. destructans* has been discovered in Northern Australia. This raises a whole series of new issues as there are now several pathogens, *K. eucalypti, K. viscidus* and *K. destructans* present in Australia that known to cause serious damage on plantation eucalypts. Recent investigations have also revealed several undescribed *Kirramyces* spp. in Northern Australia. Their impact, distribution, movement and potential for hybridization now need to be examined.

Publications arising from the current thesis

Published papers

Andjic V, Hardy GEStJ, Cortinas MN, Wingfield MJ, Burgess TI, 2007. Multiple gene genealogies reveal important relationships between *Phaeophleospora* spp. infecting *Eucalyptus* leaves. *FEMS Microbiology Letters* **268**: 22-33. (Chapter 2).

Andjic V, Barber PA, Carnegie AJ, Hardy GEStJ, Wingfield MJ, Burgess TI, 2007. Phylogenetic reassessment supports accommodation of *Phaeophleospora* and *Colletogloeopsis* from eucalypts in *Kirramyces*. *Mycological Research* **111**(10): 1184-1198 (Chapter 3).

Andjic V, Barber PA, Carnegie AJ, Pegg GS, Hardy GEStJ, Wingfield MJ, Burgess TI, 2007. *Kirramyces viscidus* sp. nov, a new eucalypt pathogen from tropical Australia is closely related to the serious leaf pathogen, *Kirramyces destructans*. *Australasian Plant Pathology* **36**(5): 478-487 (Chapter 6).

Burgess TI, Andjic V, Hardy GEStJ, Dell B, Xu D, 2006. First report of *Phaeophleospora destructans* in China. *Journal of Tropical Forest Science* **18**: 51-54 (Appendix I).

Burgess TI, Andjic V, Wingfield MJ, Hardy GEStJ, 2007. The eucalypt leaf blight pathogen *Kirramyces destructans* discovered in Australia. *Australasian Plant Pathology Disease Notes* **2**: 141-144 (Appendix II).

Conference presentations

Andjic V, Burgess IT, Hardy GEStJ, 2004. *Phaeophleospora* leaf blight diseases of plantation eucalypts in Australia; Talk presented at Asian Mycological Congress, ChaingMai, Thailand, November 2004.

Andjic V, Burgess TI, Hardy GEStJ, Wingfield MJ, 2005. True phylogenetic status of *Phaeophleospora destructans*-what is *Phaeophleospora*? Talk presented at *Mycosphaerella* Leaf Diseases of Eucalypts Workshop, 15th Australasian Plant Pathology Society Conference, Geelong, Victoria, September 2005.

Andjic V, Hardy GEStJ, Xu D, Burgess TI, Dell B, 2005. Movement of *Phaeophleospora destructions* throughout Asia; a threat to Australian native forests. Poster presented at APPS Conference, Geelong, Victoria, September 2005. (won prize for best student poster).

Andjic V, Barber PA, Hardy GEStJ, Wingfield MJ, Burgess TI, 2006. A reassessment of *Phaeophleospora* species on eucalyptus. Talk presented at the 8th International Mycological Conference, Cairns, Queensland, Australia, August 2006.

Burgess TI, Andjic V, Hardy GESTJ, Dell B, Xu D, Wingfield MJ, 2006. Movement of the devastating *Eucalyptu*s leaf and shoot pathogen *Phaeophleospora destructan*s throughout Asia. Talk presented at the 8th International Mycological Conference, Cairns, Queensland, Australia, August 2006.

Andjic V**,** Hardy GEStJ, Dell B, Wingfield MJ, Burgess TI, 2007. Movement of the devastating *Eucalyptus* leaf and shoot pathogen *Kirramyces destructans*, throughout Asia. Poster presented at IUFRO Conference, Durban, South Africa, October 2007.

Statement on authorities for species

The authorities of scientific names of pathogens and their plant hosts will not be presented in this thesis unless they form part of a taxonomic study (Chapters 3 and 6).

List of Abbreviations and terms

eucalypts	referring to species in the genera *Eucalyptus, Corymbia* and *Angophora*
MEA	malt extract
OMA	oatmeal agar
TWA	tap water agar
gL-1	gram per litre
µl	microliters
hr	hours
min	minutes
sec	second
g	acceleration gravity
diam	diameter
ha	hectares
pmol	pico mol
Taq	thermostable polymerase
CTAB	haxadecil trimethyl ammonium bromide
IAC	isoamyl alcohol
PVP	povidone iodine
TRIS	trishydroxymethylaminomethane
DNA	deoxyribonucleic acid
FIASCO	fast isolation of sequences containing repeats
AFLP	amplified fragment length polymorphism
PCR	polymerase chain reaction
ITS	internal transcribed spacer
rRNA	ribosomal ribonucleic acid
ß-tubulin	beta tubulin
EF-1α	elongation factor 1α gene
CHS	chitin synthase 1 gene
RPB2	polymerase II, subunit of ribonucleic acid
ATP-6	ATPase gene region
BLAST	**B**asic **L**ocal **A**lignment **S**earch **T**ool
PHT	partition homogeneity test
PAUP	phylogenetic analysis using parsimony
GTR	general time reversible
G	Gamma substitution model
I	Proportion of invariable site
MCMC	Markov Chain Monte Carlo

gl	phylogenetic signal
CI	consistency index
RI	retention index
bp	base pair
DAR	New South Wales, Plant Pathology Herbarium, Australia
PREM	South African national collection of fungi
CMW	Forestry and Agricultural Biotechnology Institute, Culture Collection, University of Pretoria, South Africa
NSWF	State Forests of New South Wales, Australia
CBS	Centraalbureau voor schimmelcultures, culture collection, Utrecht, the Netherlands
MUCC	Murdoch University culture collection
MURU	Murdoch University herbarium collection
BRIP	Department of Primary Industries, Brisbane, Queensland, Australia
AQIS	Australian Quarantine Inspection Service
QLD	Queensland
FNQ	far north Queensland
C-QLD	central Queensland
S-QLD	south Queensland
NSW	New South Wales
TAS	Tasmania
VIC	Victoria
NZ	New Zealand

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CHAPTER 1

Literature Review

Eucalyptus *plantations*

Eucalypts (including the genera *Eucalyptus*, *Corymbia*, *Angophora*) trees are native to Australia and are highly favoured plantation species, as they are fast growing and easy to cultivate (Turnbull 2000). Over 700 eucalypts spp. have been described in Australia (Brooker & Kleinig 1999; Brooker & Kleinig 2002; Brooker & Kleinig 2004). Beyond Australia, most eucalypt trees are exotic, except a few species in Papua New Guinea and some parts of Indonesia and Mindanao in the Philippines (Brooker & Kleinig 2004; Potts & Pederick 2000). Eucalypts are genetically diverse and have adapted to a wide climatic range and soil fertility across Australia. In Australia, forest area covers 164 million ha from which 162 million ha are native forests and woodlands and 1.6 million ha are plantation forests of which half are eucalypts (National Forestry Inventory 2004). The eucalypt plantation industry predominantly provides pulp for paper industry and saw logs for buildings (Eldridge *et al.*1994).

Plantation forestry is relatively new in Australia but is rapidly increasing in size as the harvest from native eucalypt stands is reduced and worldwide demand for paper increased (Regional Forest Agreements http://www.daff.gov.au/forestry). Most plantations are situated in southern Australia and are mostly comprised of *E. globulus*. However, some small eucalypt plantations for both timber and pulpwood trial plantations have been established in the subtropical regions of northern New South Wales and southern Queensland and are now expanding into the tropical regions of northern Queensland (Florence 1996; Carnegie *et al*. 2005; Dickinson *et al*. 2004).

Eucalypts were introduced from Australia to other parts in the world in nineteenth century and their main uses included the provision of shelter belts leaf-oil production, ornamentals and fuel. This situation changed once eucalypts were recognised as an important source of timber production (Turnbull 2000). The timber is an important source of fibre to the international paper and pulp industry in Australia and worldwide (Turnbull 2000). In the last 40 years, to meet the world's demand for pulp and paper, many commercial plantations have been established outside Australia. Most of these have been developed in the tropics and Southern Hemisphere (Eldridge *et al*. 1994). The estimated global

area of planted eucalypts was close to 17 million hectares in 2000 (Food and Agricultural Organization 2000). Of those, 8 million ha were established in India, 3.2 million ha in Brasil, 1.3 million ha in China, 0.6 million ha in South Africa, 0.6 million ha in Portugal, 0.5 million ha in Uruguay, 0.45 million ha in Vietnam, 0.45 million ha in Thailand, 0.33 million ha in Chile, 0.3 million ha in Argentina, 0.19 million ha in Philippines, and 0.13 million ha in Indonesia (Food and Agricultural Organization 2000). It is predicted that the total area of planted eucalypts will exceed 20 million ha by 2010 (Turnbull 2000). The most widely planted species are *E. globulus*, *E. pellita*, *E. urophylla*, *E. camaldulensis*, *E. grandis*, *E. tereticornis* and hybrids between these species (Turnbull 2000).

Foliar Pathogens of Eucalyptus *plantations*

Many fungi have been described from eucalypt foliage in Australia (Sankaran *et al*. 1995) but most were considered to be endemic and were not of concern in forest management as they predominantly caused diseases on native eucalypts where their impact was low (Park *et al*. 2000). This was due to balanced co-evolution between the trees and their parasites and genetic and age diversity (Heather 1967a; Burdon & Chilvers 1974; Manion 1981; Hansen 1999). However, the ecological balance of natural ecosystems can be disturbed by humans or natural disasters. Examples of human disturbance include; logging, monoculture plantations, clonal propagation, movement of infected germplasm, cuttings, and planting species on less than suitable sites (Burgess & Wingfield 2002).

Outside of Australia, eucalypts were introduced and grown as exotics. This means that these eucalypts were free of pests and diseases which are naturally associated with them in their regions of origin (Wingfield *et al*. 2001). It is generally recognised that the success of these plantations has been due to separation of these trees from their natural enemies (Wingfield 2003). This was a "honeymoon period". During this period, many species and provenance trials were established to select the best planting stock via genetic selection and breeding programmes which led to clonal forestry and monoculture practises (Cotterill & Brolin 1997; Borralho 1997). Despite the artificial barrier between the exotic plantations and their enemies, the rapid expansion of genetically uniform exotic eucalypt plantations has resulted in diseases outbreaks. Some of these outbreaks were caused by indigenous

pathogens, not found in Australia, which jumped onto eucalypts eg. *Puccinia psidii* (Walker 1983; Coutinho *et al*. 1998; Glen *et al*. 2007), *Cryphonectria cubensis* (Hodges *et al*. 1986; Myburgh *et al.* 1999), *Ceratocystis fimbriata* (Kile *et al*. 1996; Roux *et al*. 2000), and *Colletogloeopsis zuluensis* Wingfield *et al*. 1997). However, most diseases in exotic plantations are caused by pathogens that are known to infect stems and leaves of eucalypts in Australia and have been introduced from Australia with germplasm or as endophytes (Burley 1987). Among the most important of those are *Cylindrocladium* and *Mycosphaerella* species.

Cylindrocladium spp. cause destructive blights on young eucalypts and are a serious threat to eucalypts in tropical India and in equatorial regions of Brazil and Vietnam (Mohanan & Sharma 1986; Blum & Dianese 1993; Old & Yuan 1994). In Australia, a *Cylindrocladium scoparium* caused the death of young eucalypts in nurseries in northern New South Wales (Keirle 1981), but was not considered as a threat to forests in NSW as the disease can be controlled in container nurseries by cultural methods and systemic fungicides thus can not be transferred from the nursery to the plantation.

Mycosphaerella spp. and their anamorphs are one of the most common causes of foliar disease that affect exotic plantations throughout the world (Carnegie *et al*. 1997; Park *et al*. 2000; Barber *et al*. 2003; Maxwell *et al*. 2003). Their incidence and severity is increasing as the areas under cultivation expand (Park *et al*. 2000; Maxwell, *et al*. 2003). There are over 100 *Mycosphaerella* species. Many *Mycosphaerella* spp. or their anamorphs have broad host and geographic range such as *Phaeophleospora epicoccoides,* while others are more specific like *M. ohnowa* which is known to cause disease on *E. grandis* and *E. smithii* in South Africa (Crous *et al*. 2004). The most destructive *Mycosphaerella* spp. are *Mycosphaerella cryptica*, *M. nubilosa* and *Phaeophleospora destructans* (Park *et al*. 2000).

Mycosphaerella spp. occurring on eucalypts are generally considered to be eucalypt-specific, but many new species were first described from countries other than Australia, due to their impact on exotic eucalypt plantations (Crous *et al*. 2004; Crous *et al*. 2006; Hunter *et al*. 2004). Less than half of the *Mycosphaerella* spp. or their anamorphs, shown to be pathogens outside Australia, have been

found in Australia. However, this does not mean they are absent from endemic eucalypts in Australia and as the plantation estate grows, it is likely that more of these *Mycosphaerella* spp. will be found. Indeed, *M. fori* and *M. heimii,* first described from South Africa (Hunter *et al*. 2004), Madagascar and Indonesia (Crous 1998), have been recently found causing disease in Australia (Jackson *et al*. 2005, Whyte *et al*. 2005). There are a few reports of non-eucalypt *Mycosphaerella* pathogens causing disease on eucalypts. For example, *Mycosphaerella citri* originally described from citrus was recently isolated from *E. camaldulensis* in Vietnam (Burgess *et al*. 2007a).

Phaeophleospora *spp. on* Eucalyptus

Phaeophleospora is an anamorph genus commonly causing leaf and shoot blight diseases of eucalypts (Crous 1998; Crous *et al*. 2001; Maxwell *et al*. 2003; Crous *et al*. 2004). Some *Phaeophleospora* spp. Have a *Mycosphaerella* teleomorph. Taxonomically *Phaeophleospora* species belong to class; *Ascomycota*, *Dothideomycetes*, *Dothideomycetidae*, *Capnodiales*, *Mycosphaerellaceae*, genus; *Phaeophleospora*.

The type specimen for the genus is *Phaeophleospora eugeniae* isolated from *Eugenia uniflora*. This genus was introduced to accommodate the dark form of *Phleospora* (*Phloeospora*) by Rangel (1916). Up to 1997 most taxonomists regarded the monotypic genus *Phaeophleospora* as a *nomen dubium* because the mode of conidiogenesis and the form of the conidia were not documented in the type description (Sutton 1977). In 1997, Crous *et al*. (1997a) redescribed *Phaeophleospora eugeniae* based on the collection and designation of a neotype. In their study, it was concluded that *P. eugeniae* resembled species residing in *Kirramyces,* and *Kirramyces* was reduced to synonymy under the older name *Phaeophleospora*. Thus the genus was expanded from one to eight species; *P. eugeniae*, *P. destructans*, *P. epicoccoides*, *P. eucalypti*, *P. hebes*, *P. lilianiae*, *P. proteae*, *P. phormii*. Later, *Stagonospora delegatensis* was recognized as a species similar to *K. eucalypti* and was also transferred to *Phaeophleospora* (Crous 1998).

Phaeophleospora species causing leaf blight disease of eucalypt include; *Phaeophleospora destructans*, *Phaeophleospora delegatensis*, *Phaeophleospora epicoccoides*, *Phaeophleospora eucalypti*, *Phaeophleospora lilianiae*, *Phaeophleospora toledana*, *Phaeophleospora delegatensis*.

Rarely encountered **Phaeophleoaspora** ***spp.***

Phaeophleospora delegatensis, *P. eugeniae*, *P. lilianiae* and *P. toledana* are rarely encountered species. The teleomorph of *P. eugeniae* has not been seen. *Phaeophleospora eugeniae* causes leaf spots on *Eugenia uniflora*, but is not considered as important pathogen, nor is it known as a pathogen of eucalypts. *Phaeophleospora toledana* is an anamorph of *M. toledana* named after the location from which was collected Toledo, Spain (Crous *et al*. 2004).

Phaeophleospora delegatensis is an anamorph of *Mycosphaerella delegatesis* isolated from the leaves of *E. delegatensis* and *E. obliqua* from Victoria, Australia where it occasionally causes premature defoliation if the infection levels are severe. Due to poor survival, this fungus has never been cultured, and to date there is no DNA sequence data for this fungus.

The teleomorph of *P. lilianiae* has not been seen. This fungus has been found only on yellow bloodwood *Corymbia* (*Eucalyptus*) *eximia*, that occurs naturally only on the coast of New South Wales mainly on poorer sandstones soils (Chippendale 1988). *Phaeophleospora lilianie* was described as very similar to *P. epicoccoides*, but differs by having shorter and cylindrical conidia with one to three septa (Walker *et al.* 1992). The fungus has not been stored in culture, and to date there are no DNA sequence data.

Phaeophleospora delegatensis, *P eugeniae*, *P. lilianiae* and *P. toledana* are not considered serious pathogens. Among all known *Phaeophleospora* species from eucalypts, the most pathogenic and threatening are *P. destructans, P. epicoccoides* and *P. eucalypti* and these will be discussed further.

Phaeophleospora destructans

This pathogen was first described from 1-3 year-old *E. grandis* in Indonesia in 1996 and has subsequently been found in Thailand, China, Vietnam and Timor (Old *et al*. 2003a; Old *et al*. 2003b; Burgess *et al*. 2006a). Besides *E. grandis*, this pathogen has been found on leaves of clones of *Eucalyptus grandis* x *E. camadulensis* and *E. urophylla* (Old *et al*. 2003b).

Based on phylogenetic data, *P. destructans* is an anamorph of *Mycosphaerella* closely related to *P. eugeniae* (Crous *et al.* 2001). It is an aggressive and often devastating pathogen that causes distortion of infected leaves and blight of young leaves, buds and shoots (Wingfield *et al*. 1996). The symptoms include large sub-circular light brown leaf spots with a diffuse border and red brown margin which are present on both surfaces. Infected leaves are malformed, delimited by the veins that commonly display purple discolouration on older leaves (Wingfield *et al*. 1996).

The origin, biology, host range, epidemiology, genetics and transmission of *P. destructans* is unknown. It is a highly virulent pathogen known to affect some exotic eucalypts and hybrids in Asia (Burgess *et al*. 2006a; Old *et al*. 2003b; Wingfield *et al*. 1996). The susceptibility of other Australian endemic eucalypts is unknown. Thus, the importance of *P. destructans* to Australian native forests and the forestry industry is unknown. This fungus threatens the biosecurity of Australia's eucalypts and is currently on the Biosecurity watch list for eucalypt pathogens (http://www.daff.gov.au).

Phaeophleospora epicoccoides

Phaeophleospora epicoccoides is an anamorph of *Mycosphaerella suttonii* (Crous *et al*. 1997b). The teleomorph, *M. suttonii* has been described from Northern Sumatra, Indonesia (Crous *et al*. 1997b) but is rarely seen in nature or culture. It is one of the most widely distributed foliar pathogens on *Eucalyptus* spp. in the world has been reported from South Africa (Crous *et al*. 1988), Malawi (Chipompha 1987), Australia (Cooke 1889), Brazil (Ferreira 1989), India (Padaganur & Hiremath 1973), Thailand and Vietnam (Sharma 1994; Old & Yuan 1994), New Zealand (Dick 1982), Italy (Belisario 1993), and Chile, China (Simpson *et al*. 2005), Colombia, Portugal, and Taiwan (Wingfield, pers. comm.).

Phaeophleospora epicoccoides primarily occurs on species of subgenus *Symphyomyrtus* (Heather 1965; Crous *et al*. 1988) including; *E. bicostata, E. camaldulensis, E. delegatensis*, *E. grandis, E. globulus, E. platypus, E. saligna, E. tereticornis* (Sankaran *et al*. 1995). *Phaeophleospora epicoccoides* mostly infects mature leaves, but has also been found on some eucalypts species in nursery seedlings (Walker 1962). The symptoms caused by *P. epicoccoides* are variable worldwide and depend on host species and stage of development of infection, but most common are purple spots on leaves, necrotic lesions delimited by veins and presence of spore masses and conidia (Dick 1982; Old & Yuan 1999; Walker, 1962; Walker *et al*. 1992). Infection first appears on mature leaves and spots are on both sides of the leaf, and gradually progresses upward in the crown. Late in the season spots occur on younger leaves and all mature leaves drop. If the infections are severe, premature defoliation occurs, which then affects growth and vigour of seedlings (Carnegie 2007a).

Phaeophleospora epicoccides was one of first foliar pathogens of eucalypts to be studied in detail (Heather 1965, 1967a, 1967b; Nichol *et al*. 1992 a, 1992b). Various studies have shown that orientation of the leaf lamina, hydrophobicity, site preparation and fertilisation play an important role in disease susceptibility (Heather 1965, Nichol *et al*. 1992b).

Phaeophleospora epicoccides was serious in South Africa, where it has caused seedling mortality and complete defoliation of mature trees in plantations (Knipscheer *et al.* 1990; Nichol *et al.* 1992a; 1992b). During forest health surveys between 1996-2005 in New South Wales, this fungus was found to cause severe defoliation of *E. grandis* and *E. grandis* x *E. camaldulensis* plantations (Carnegie 2007b). While thought that this pathogen in Australia was limited only to the eastern states, it has been recently found on *E. grandis* (Jackson, pers. comm.), *E. diversicolor* (Burgess pers. comm.) and *E. rudis* (Barber pers. comm.) in Western Australia.

Phaeophleospora eucalypti

Like *P. destructans*, the teleomorph of *Phaeophleospora eucalypti* has never been seen. *Phaeophleospora. eucalypti* was first described from fading leaves of a *Eucalyptus* sp. collected from Oakleight, Victoria, Australia in 1884 (Cooke 1889). It is known as a leaf parasite of native species of eucalypts in Australia (Hood *et al.* 2002a, 2000b) and has been found in plantations of *E. nitens* and *E. globulus* in southern New South Wales, Victoria (Gippsland) and Tasmania (Yuan 1999). *Phaeophleospora eucalypti* was introduced to New Zealand via infected material, where the damage caused by the fungus resulted in complete defoliation of juvenile leaves of *E. nitens* (Dick 1982). Besides *E. nitens* and *E. globulus*, *P. eucalypti* has been found on *E. camaldulensis, E. cephalocarpa*, *E. cypellocarpa*, *E. dalrympleana, E. grandis, E. gunnii, E. ovata, E. punctata, E. platypus, E. parvula E. tereticornis, E. viminalis* (Park *et al*. 2000; Barber *et al*. 2003) and more recently on *E. grandis* x *E. urophylla*, *E. dunnii*, *E. moluccana* and *E. longirostrata*, *E. scorparia* and *E. smithii* (Carnegie 2007a).

Phaeophleospora eucalypti mostly infects young leaves with symptoms first appearing in spring and ends with defoliation. Symptoms caused by *P. eucalypti* include; blotchy, necrotic lesions, that are rounded or delimited by veins turning pale yellow and carmine red before becoming necrotic (Heather 1961; Dick 1982; Gadgil & Dick 1983). Recently, it has become a very serious pathogen in subtropical plantations in eastern Australia (Carnegie 2007a, G. Pegg, pers. comm.). *Phaeophleospora eucalypti* is native to Australia, but the origin, biology, epidemiology, genetics and transmission of this pathogen are unknown and need to be explored.

Aims

This study will focus on *Phaeophleospora* spp. from eucalypts, with an emphasis on *P. destructans* and *P. eucalypti* and will:

1. determine the morphological and phylogenetic relationship of worldwide collections of *Phaeophleospora* spp. from eucalypts including the type specimens,

2. develop species-specific primers and microsatellites markers for detecting *P. destructans* and *P. eucalypti* that can then be applied *in vitro* and *in vivo*,

3. examine the genetics and gene flow of worldwide collections of *P. destructans* and determine its source of origin,

4. examine the genetics and gene flow of *P. eucalypti* from Australia and New Zealand and determine its source of origin and the pathway of movement between plantations, and

5. describe new *Phaeophleosporas* species occurring on eucalypts.

Chapter 2

Multiple gene genealogies reveal important relationships between species of *Phaeophleospora* infecting *Eucalyptus* leaves

Published as: Andjic V, Hardy GEStJ, Cortinas MN, Wingfield MJ, Burgess TI, 2007. Multiple gene genealogies reveal important relationships between species of *Phaeophleospora* infecting *Eucalyptus* leaves. *FEMS Microbiology Letters*, **268**: 22-33.

Minor authors' contributions: Burgess TI and Hardy GEStJ were supervisors, Cortinas MN provided some sequence data, Wingfield MJ provided some isolates and financial support.

ABSTRACT

The majority of *Eucalyptus* are native to Australia, but world-wide there are over 3 million ha of exotic plantations, especially in the tropics and sub-tropics. Of the numerous known leaf diseases, three species of *Phaeophleospora* can cause severe defoliation of young *Eucalyptus*; *P. destructans, P. eucalypti* and *P. epicoccoide*s. *Phaeophleospora destructans* has a major impact on seedling survival in Asia and has not, as yet, been found in Australia where it is considered a serious threat to the biosecurity of native eucalypts. It can be difficult to distinguish *Phaeophleospora* spp. based on symptoms and micromorphology and an unequivocal diagnostic tool for quarantine purposes would be useful. In this study we have constructed a multiple gene genealogy of these *Phaeophleospora* species and designed specific primers to detect their presence from leaf samples. The phylogenetic position of these *Phaeophleospora* species within *Mycosphaerella* was established. They are closely related to each other and to other important *Eucalyptus* pathogens, *M. nubilosa*, *M. cryptica* and *Colletogloeopsis zuluensis.* The specific primers developed can now be used for diagnostic and screening purposes within Australia.

INTRODUCTION

Eucalyptus species are highly favoured for the establishment of plantations. This is due to their rapid growth, ease of cultivation and their adaptation to a wide variety of different growing conditions (Turnbull 2000). The timber of these trees is an important source of fibre for the international paper and pulp industry (Turnbull 2000). In Australia, plantation forestry is rapidly increasing in size (National Forestry Inventory 2004) and a number of fungal foliar pathogens have been reported to impact negatively on yields of these plantations. Among the most important of these pathogens are *Mycosphaerella* spp. (Barber *et al.* 2003; Carnegie *et al.* 1997; Maxwell *et al.* 2003; Park *et al.* 2000) and their incidence and severity is increasing as the areas under cultivation expand (Maxwell *et al.* 2003; Park *et al.* 2000).

Phaeophleospora Rangel is an anamorph genus assigned to some species of *Mycosphaerella* (Crous 1998; Crous *et al.* 2004; Crous *et al.* 2001; Maxwell *et al.* 2003). Six species are known to cause disease on leaves of *Eucalyptus* species. These are *P. epicoccoides* (Cooke & Massee) Crous, Ferreira & Sutton, *P. destructans* (M.J. Wingf & Crous) Crous, Ferreira & Sutton, *P. eucalypti* (Cooke & Massee) Crous, Ferreira & Sutton, *P. lilianie* (Walker, Sutton & Pascoe) Crous, Ferreira & Sutton, *P. delegatensis* R.F. Park & Keane (Crous 1998) and the recently described *P. toledana* Crous & G. Bills (Crous *et al.* 2004). Of these species, *P. epicoccoides, P. destructans* and *P. eucalypti* are considered important pathogens (Park *et al.* 2000). *Phaeophleospora lilianie* has been found only on yellow bloodwood (*Corymbia eximia*) in New South Wales and little is known regarding its importance (Chippendale 1988). *Phaeophleospora delegatensis* is the anamorph of *Mycosphaerella delegantesis* (Park & Keane 1984) isolated from the leaves of *E. delegantensis* and *E. obliqua* in Australia. It occasionally causes premature defoliation if the infection levels are severe. Both *P. liliane* and *P. delegatensis* have poor survival in culture and they have never been successfully stored. *Phaeophleospora toledana* is the anamorph of *Mycosphaerella toledana* (Crous *et al.* 2004) named for its location of origin and it is not considered as a serious leaf pathogen.

Phaeophleospora destructans is an aggressive and often devastating pathogen that causes distortion of infected leaves and blight of young leaves, buds and shoots (Wingfield *et al.* 1996). This pathogen was

first discovered in Indonesia in 1996 and has subsequently been found in Thailand, China, Vietnam and Timor (Burgess *et al.* 2006a; Old *et al.* 2003a, 2003b). While most *Phaeophleospora* spp. infecting *Eucalyptus* leaves are known from Australia, it is intriguing that *P. destructans,* the most pathogenic of these fungi has not been found in this country. Thus, the potential impact that *P. destructans* might have on native eucalypt forests is unknown, but of concern.

Phaeophleospora epicoccoides is the anamorph of *M. suttonii* (Crous *et al.* 1997b) and it occurs worldwide infecting almost all *Eucalyptus* species (Sankaran *et al.* 1995). This species is well known on native *Eucalyptus* spp. in Australia and it has most likely been spread to other countries with germplasm used to establish plantations. *Phaeophleospora epicoccoides* is a relatively weak pathogen that typically infects older leaves and stressed trees (Knipscheer *et al.* 1990). *Phaeophleospora eucalypti,* a native pathogen in Australia, has in the past resulted in complete defoliation of juvenile leaves of *E. nitens* in New Zealand, the only country where it is known to have been introduced (Dick 1982; Hood *et al*. 2002a, 2002b).

The appearance and severity of lesions on *Eucalyptus* leaves are generally used to recognise the species of *Phaeophleospora* responsible for disease. However, depending on host and climate, the symptoms associated with infection by *P. epicoccoides*, *P. eucalypti* and *P. destructans* can be almost identical (Figure 1) and incorrect diagnosis is a common problem. In addition, identification of *P. eucalypti* and *P. destructans* based on conidial morphology can be difficult because spore size varies depending on host species. A simple and accurate molecular diagnostic technique to distinguish between these important species would compliment traditional morphological diagnosis.

The aim of this study was to construct multiple gene genealogies for *Phaeophleospora epicoccoides*, *P. destructans* and *P. eucalypti*, the most common and destructive species occurring on eucalypts. Thus, partial sequence for six protein coding genes were generated to elucidate the phylogenetic relationships between these *Phaeophleospora* species. Following the construction of the phylogenies, species specific primers were then designed for diagnostic purposes.

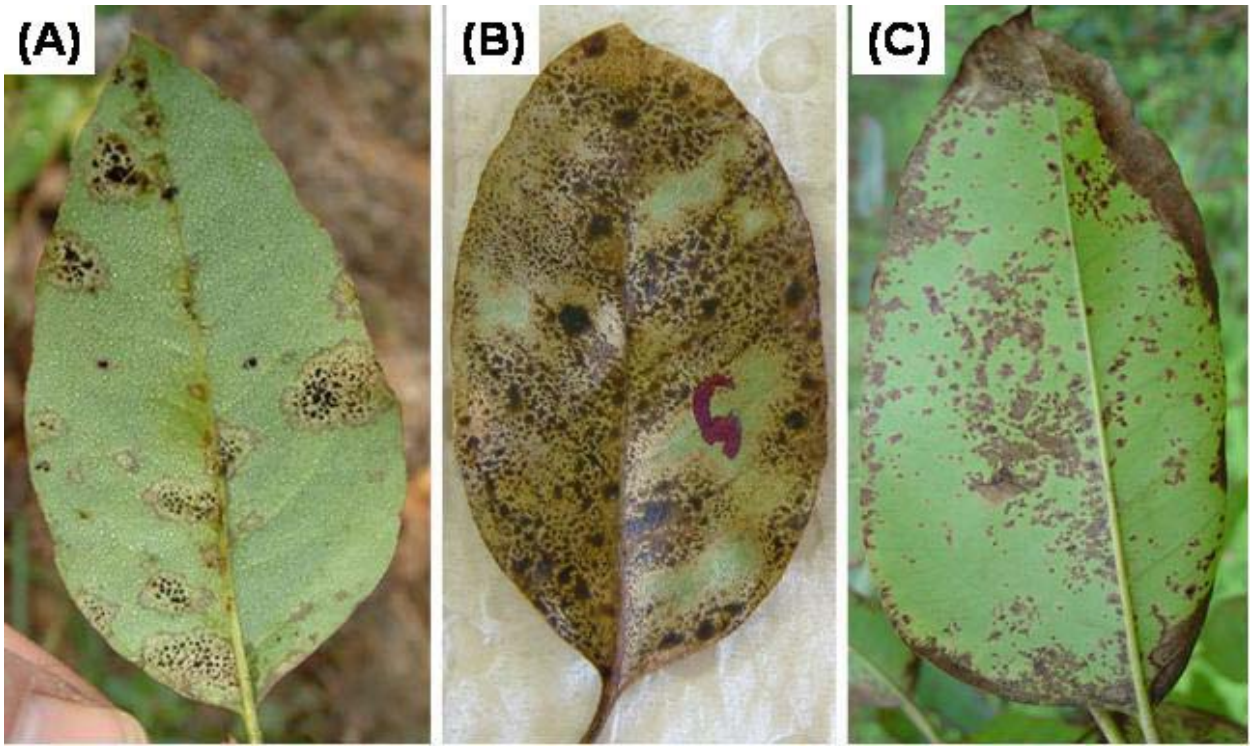


Figure 1. Comparison of symptoms produced on juvenile *Eucalyptus grandis* leaves infected with (A) *Phaeophleospora destructans*, (B) *Phaeophleospora eucalypti* and (C) *Phaeophleospora epicoccoides* showing the similarity of symptoms associated with these fungi.

MATERIALS AND METHODS

Fungal isolates

Isolates were obtained under a dissecting microscope by collecting conidia exuding from single pycnidia, on the tip of a sterile needle. These were transferred onto 2% Malt Extract Agar (MEA) containing Streptomycin 150µg/ml (Sigma-Aldrich, Sydney, Australia) in a single spot and allowed to hydrate for 5 min. Spores were then streaked using a sterile needle and single spores immediately transferred to new MEA plates. Cultures were grown at 25°C for 2 weeks and then transferred to fresh MEA plates. Cultures were maintained on 2% MEA in tubes at 20°C. Isolates made for this study were compared with those of other closely related species (Table 1). All isolates are maintained in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa or the Murdoch University culture collection (MUCC), Perth, Western Australia.

DNA Extraction

Isolates were grown on 2% MEA at 20°C for 4 weeks and the mycelium was harvested, frozen in liquid nitrogen, ground to a fine powder and genomic DNA extracted using a hexadecyl trimethyl ammonium bromide (CTAB) protocol from (Graham *et al.* 1994) modified by the addition of 100 μg/ml Proteinase K and 100 μg/ml RNAse A to the extraction buffer.

PCR amplification

This study included partial amplification of the 18S gene, the complete internal transcribed spacer region 1, the 5.8S rDNA gene and the complete internal transcribed spacer region 2 and the 5' end of the 26S (large subunit) rDNA (ITS), part of the β-tubulin gene region (βt), part of elongation factor 1α gene (EF-1α), part of Chitin synthase 1 gene (CHS), part of the RNA polymerase II subunit (RPB2) and part of ATPase gene (ATP-6). Primers used for amplification of these regions are listed in Table 2. The PCR reaction mixture (25 μl), PCR conditions and visualisation of products were as described previously (Cortinas *et al.* 2006b) except that 1 U of Taq polymerase (Biotech International, Needville, Texas, USA) was used in each reaction. For failed amplifications, the Mg concentration was increased to 4 mM, and primer concentration to 0.9 *p*mol and the following PCR conditions were used; 7 min at 94°C, followed by 35 cycles of 1 min at 94° C, 1 min at 45° C, 2 min at 72° C and final elongation step of 10 min at 72° C. RPB2 degenerate primers were tested at a range of temperatures, but failed to amplify the DNA of some representative isolates. Therefore, two successful amplicons were sequenced and primers re-designed and named RPB2-myco-F and RPB2-myco-R (Table 2). The PCR products were purified with Ultrabind®DNA purification kit (MO BIO Laboratories, Solana Beach, California, USA) following the manufacturer instructions. Amplicons were sequenced as described previously (Burgess *et al.* 2005).

Table 1. Species and isolates considered in the phylogenetic study.

Culture no.[1]	Teleomorph	Anamorph	Host	Location	Collector	GenBank Accession no's[2]			
						ITS	ß-tubulin	EF-1α	CHS
STE-U 1454 CMW 5351		*Phaeophleospora eugeniae*	*Eugenia uniflora*	Brazil	MJ Wingfield	AF309613 **DQ632710**			
STE-U1366 CMW 5219		*P. destructans*	*Eucalyptus grandis*	Sumatra, Indonesia	MJ Wingfield	AF309614 **DQ632699**			
CMW 7127		*P. destructans*	*Eucalyptus* sp.	Sumatra, Indonesia	MJ Wingfield	**DQ632698**			
CMW 19906		*P. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	**DQ632700**			
CMW 22553		*P. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	**DQ632667**	**DQ632625**	**DQ632732**	**DQ632646**
CMW 17918		*P. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	**DQ632666**	**DQ632624**	**DQ632731**	**DQ632645**
CMW 19832		*P. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	**DQ632665**	**DQ632623**	**DQ632730**	**DQ632644**
CMW 17919		*P. destructans*	*E. urophylla*	Guangzhou, China	TI Burgess	**DQ632701**	**DQ632622**	**DQ632729**	**DQ632643**
MUCC 433		*P. eucalypti*	*E. nitens*	Victoria, Australia	PA Barber	**DQ632661**	**DQ632631**	**DQ632726**	**DQ632650**
CMW 17915		*P. eucalypti*	*E. nitens*	Victoria, Australia	PA Barber	**DQ632664**	**DQ632626**	**DQ632727**	**DQ632653**
MUCC 432		*P. eucalypti*	*E. grandis x E. tereticornis*	New South Wales	AJ Carnegie	**DQ632660**	**DQ632627**	**DQ632724**	**DQ632648**
MUCC 434		*P. eucalypti*	*E. grandis x E. tereticornis*	New South Wales	AJ Carnegie	**DQ632662**	**DQ632632**	**DQ632728**	**DQ632651**
CMW 17917		*P. eucalypti*	*E. grandis x E. tereticornis*	New South Wales	AJ Carnegie	**DQ632711**	**DQ632630**	**DQ632725**	**DQ632649**
MUCC 435		*P. eucalypti*	*E. grandis x E. camaldulensis*	Queensland	AJ Carnegie	**DQ632663**	**DQ632629**	**DQ632723**	**DQ632652**
CMW 17916		*P. eucalypti*	*E. grandis x E. camaldulensis*	Queensland	AJ Carnegie	**DQ632659**	**DQ632628**	**DQ632722**	**DQ632647**
CMW 11687		*P. eucalypti*	*E. nitens*	New Zealand	M Dick	DQ240001	**DS890168**	DQ235115	**DQ890167**
NZFS85C/23		*P. eucalypti*	*E. nitens*	New Zealand	M Dick	AY626988			
NZFS85C/1		*P. eucalypti*	*E. nitens*	New Zealand	M Dick	AY626987			
MUCC 422	*M. suttonii*	*P. epicoccoides*	*E. grandis x E. camaldulensis*	Queensland	G Hardy	**DQ632656**			
MUCC 424	*M. suttonii*	*P. epicoccoides*	*E. grandis x E. camaldulensis*	Queensland	G Hardy	**DQ632703**	**DQ632617**	**DQ632712**	**DQ632633**
MUCC 428	*M. suttonii*	*P. epicoccoides*	*E. grandis x E. camaldulensis*	Queensland	TI Burgess	**DQ632707**	**DQ632618**	**DQ632717**	**DQ632638**
MUCC 430	*M. suttonii*	*P. epicoccoides*	*E. grandis*	Queensland	G Whyte	**DQ632708**			
MURU 327	*M. suttonii*	*P. epicoccoides*	*E. globulus*	Western Australia	S Jackson	**DQ632702**	**DQ632619**	**DQ632716**	**DQ632639**
MUCC 426	*M. suttonii*	*P. epicoccoides*	*E. globulus*	Western Australia	S Jackson	**DQ632704**	**DQ632620**	**DQ632715**	**DQ632637**
CMW 22482	*M. suttonii*	*P. epicoccoides*	*E. grandis*	Sumatra, Indonesia	PA Barber	**DQ632658**	**DQ632621**	**DQ632719**	**DQ632636**
MUCC 425	*M. suttonii*	*P. epicoccoides*	*E. grandis*	New South Wales	TI Burgess	**DQ632655**	**DQ632613**	**DQ632713**	**DQ632634**
MUCC 429	*M. suttonii*	*P. epicoccoides*	*E. grandis*	New South Wales	TI Burgess	DQ530226			

Culture no.[1]	Teleomorph	Anamorph	Host	Location	Collector	GenBank Accession no's[2]			
						ITS	ß-tubulin	EF-1α	CHS
MUCC 431	*M. suttonii*	*P. epicoccoides*	*E. grandis*	New South Wales	TI Burgess	DQ530227			
CMW 22484	*M. suttonii*	*P. epicoccoides*	*E. urophylla*	China	TI Burgess	**DQ632705**	**DQ632616**	**DQ632714**	**DQ632635**
CMW 22486	*M. suttonii*	*P. epicoccoides*	*E. urophylla*	China	TI Burgess	**DQ632706**	**DQ632615**	**DQ632720**	**DQ632642**
CMW 17920	*M. suttonii*	*P. epicoccoides*	*E. urophylla*	China	TI Burgess	**DQ632654**	**DQ632612**	**DQ632721**	**DQ632641**
CMW 22483	*M. suttonii*	*P. epicoccoides*	*E. grandis*	Indonesia	PA Barber	**DQ632709**			
CMW 5348, STE-U 1346	*M. suttonii*	*P. epicoccoides*	*Eucalyptus* sp.	Indonesia	MJ Wingfield	AF309621	DQ240117	DQ240170	DQ890166
SA12	*M. suttonii*	*P. epicoccoides*	*E. sp.*	South Africa	MN Cortinas	**DQ632657**	**DQ632614**	**DQ632718**	**DQ632640**
STE-U 10840, CPC 10840	*M. toledana*	*P. toledana*	*E. globulus*	Spain	PW Crous	AY725580			
CBS 113313, CMW 14457	*M. toledana*	*P. toledana*	*E. globulus*	Spain	PW Crous	AY725581	**DQ658235**	DQ235120	**DQ658226**
AMR 051	*M. nubilosa*		*E. globulus*	Western Australia	A Maxwell	AY509777			
AMR 057	*M. nubilosa*		*E. globulus*	Western Australia	A Maxwell	AY509778			
CMW 11560	*M. nubilosa*		*E. globulus*	Tasmania	A. Milgate	**DQ658232**	**DQ658236**	DQ240176	**DQ658230**
CMW 6211	*M. nubilosa*		*E. nitens*	South Africa	G Hunter	AF449094			
CMW 9003	*M. nubilosa*		*E. nitens*	South Africa	G Hunter	AF449099			
AMR 118	*M. cryptica*	*Colletogloeopsis nubilosum*	*E. globulus*	Western Australia	A Maxwell	AY509753			
AMR 115	*M. cryptica*	*C. nubilosum*	*E. globulus*	Western Australia	A Maxwell	AY509754			
CMW 3279	*M. cryptica*	*C. nubilosum*	*E. globulus*	Australia	AJ Carnegie	AY309623	**DQ658234**	DQ235119	**DQ658225**
CMW 4915		*C. zuluensis*	*E. grandis*	South Africa	MJ Wingfield	AY244421			
CBS 117262, CMW 7449		*C. zuluensis*	*E. grandis*	South Africa	L Van Zyl	DQ240021	DQ240102	DQ240155	**DQ658224**
CBS 113399, CMW 13328		*C. zuluensis*	*E. grandis*	South Africa	L Van Zyl	DQ240018	**DQ658233**	DQ240172	**DQ658223**
CBS 110499, CMW 13704	*M. ambiphylla*	*Phaeophleospora* sp.	*E. globulus*	Western Australia	A Maxwell	AY150675	DQ240116	DQ240169	**DQ658229**
STE-U 784	*M. molleriana*	*C. molleriana*	*Eucalyptus* sp.	USA		AF309619			
CMW 4940, CPC1214	*M. molleriana*	*C. molleriana*	*Eucalyptus* sp.	Portugal	MJ Wingfield	DQ239969	DQ240115	DQ240168	**DQ658228**
A/1/8	*M. vespa*	*Coniothyrium ovatum*	*Eucalyptus* sp.	Tasmania	A Milgate	AY045499			
CMW 11588	*M. vespa*	*Co. ovatum*	*E. globulus*	Tasmania	A Milgate	DQ239968	DQ240114	DQ240167	**DQ658227**
CMW 6210	*M. vespa*	*Co. ovatum*	*E. globulus*	New South Wales	MJ Wingfield	AF449095			
CBS 110906		*Coniothyrium* sp.	*E. cladocalyx*	South Africa	PW Crous	AY725513			
CBS 111149		*Coniothyrium* sp.	*E. cladocalyx*	South Africa	PW Crous	AY725514			
CBS 111149		*Coniothyrium* sp.	*E. cladocalyx*	South Africa	PW Crous	AY725514			

Culture no.[1]	Teleomorph	Anamorph	Host	Location	Collector	GenBank Accession no's[2]			
						ITS	ß-tubulin	EF-1α	CHS
CBS 113621		*Coniothyrium* sp.	*E. cladocalyx*	South Africa	PW Crous	AY725515			
CBS 116427		*Coniothyrium* sp.	*Eucalyptus* sp.	South Africa	PW Crous	AY725516			
CPC 18		*Coniothyrium* sp.	*E. cladocalyx*	South Africa	PW Crous	AY725517			
CBS 116428		*Coniothyrium sp.*	*Eucalyptus* sp.	South Africa	P.W. Crous	AY725518			
CBS 113265, CMW 13333	*M. punctiformis*	*Ramularia endophylla*	*Quercus robor*	Netherlands		AY490763			
CMW 9091	*M. marksii*		*Eucalyptus* sp.	South Africa	G Hunter	AF468871			
STE-U 796, CBS 680.95	*M. africana*		*Eucalyptus* sp.	South Africa	PW Crous	AF173314			
STE-U 1084	*M. keniensis*		*Eucalyptus* sp.	Kenya		AF173300			
CBS 110500, AMR 221	*M. aurantia*		*E. globulus*	Western Australia	A Maxwell	AF509743			
CBS 110969, STE-U1106	*M. colombiensis*	*Pseudocercospora colombiensis*	*Eucalyptus* sp.	Colombia	MJ Wingfield	AF309612			
CBS 110503, AMR 251	*M. parva*		*E. globulus*	Western Australia	A Maxwell	AF509782			
NZs	*M. suberosa*				A. Milgate	AY045503			
CBS 110949	*M. ohnowa*		*E. grandis*	South Africa	MJ Wingfield	AY725575			
STE-U 1225	*M. ellipsoidea*	*Uwebraunia ellipsoidea*	*Eucalyptus* sp.	South Africa	MJ Wingfield	AF173303			
CMW 9098	*M. ellipsoidea*	*U. ellipsoidea*	*Eucalyptus* sp.	South Africa	MJ Wingfield	AF468874			
CMW 7774	*Botryosphaeria obtusa*	*Diplodia seriata*	*Ribes* sp.	New York, USA	B. Slippers	AY236953			
CMW 7773	*Neofusicoccum ribis*		*Ribes* sp.	New York, USA	B. Slippers	AY236936	AY808170	AY236878	**DQ658231**

[1] Designation of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; STE-U = Stellenboch University, South Africa; MUCC = Murdoch University, Perth, Western Australia, [2] Sequences in bold were obtained during this study

Table 2. Primer sets and annealing temperature used to amplify *Phaeophleospora* spp.

Region	Oligos	Oligo Sequence (5'-3')	Amplicon size (bp)	AT (ºC)	Reference
ITS	ITS-1F ITS-4	CTTGGTCATTTAGAGGAAGTAA TCCTCCGCTTATTGATATGC	600	50	(Gardes & Bruns 1993)
ITS	ITS-3 ITS-4	GTATCGATGAAGAACGCAGC TCCTCCGCTTATTGATATGC	250	55	(White *et al.* 1990)
β-tubulin	βt2a βt2a	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	680	45 55	(Glass & Donaldson 1995)
EF-1α	EF1-728F EF1-986R	CATCGAGAAGTTCGAGAAGG TACTTGAAGGAACCCTTACC	350	45-55	(Carbone & Kohn 1999)
CHS	CHS-79F CHS-354R	TGTGGGCAAGGATGCTTGGAAGAAG TGGAAGAACCATCTGTGAGAGTTG	300	55	(Carbone & Kohn 1999)
RPB2	RPB2-F RPB2-R	CAAGGTCTTCACAGATGC CCCATRGCTTGYTTRCCCAT	1400	45-55	(Liu *et al.* 1999)
RPB2myco	RPB2myco-F RPB2myco-R	CAAGGTCTTCACAGATGC CAGGATGAATCTCGCAATG	650	50-55	This study
ATP6	ATP6-1 ATP6-2	ATTAATTSWCCWTTAGAWCAATT TAATTCTANWGCATCTTTAATRTA	600	45	(Kretzer & Bruns 1999)
β-tubulin (*P.destructans*)	Pdβt-F Pdβt-R	GTAACCAAATCGGTGCTGCT CAAAGTGGCTGCTCCGGGCG	198	62	This study
EF-1α (*P.destructans*)	Pd-EF-F Pd-EF-R	CGAGAAGTTCGAGAAGGTCAG GCGAGGGCTCTGTCGAAG	204	62	This study
β-tubulin (*P. eucalypti*)	Pey-βt-F Pey-βt-R	GTAACCAAATCGGTGCTGCT GAGTACAAGTGGCTGCTTAG	203	62	This study
EF-1α (*P. eucalypti*)	Pey-EF-F Pey-EF-R	CGAGAAGTTCGAGAAGGTCAG CTCTATCTGAAAGTCTTGGC	229	62	This study
β-tubulin (*P.epicoccoides*)	Pep-βt-F Pep-βt-R	CGACGGCTCAGGCGTGTATG GCGTTAGTGGTGTTGCTTGA	218	62	This study
EF-1α (*P.epicoccoides*)	Pep-EF-F Pep-EF-R	CCTACACACCCGCTGGTTAC CGGCGATCCTCCATAATCT	173	62	This study

Base codes: R (AG), Y (CT), N (AGCT), S (GC), W (AT)

Phylogenetic analyses

In order to compare *Phaeophleospora* isolates used in this study with other closely related species, additional sequences were obtained from GenBank (Table 1). Sequence data were assembled using Sequence Navigator version 1.01 (Perkin Elmer, Melbourne, Australia) and aligned in ClustalX (Thompson *et al.* 1997) Manual adjustments were made visually by inserting gaps where necessary. All sequences obtained in this study have been deposited in GenBank and accession numbers are shown in Table 1.

The initial analysis was performed on an ITS dataset alone and subsequent analyses was performed on a combined dataset of ITS, βt, CHS and EF-1α sequence, after a partition homogeneity test (PHT) had been performed in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2003) to determine whether sequence data from the four separate gene regions were statistically congruent (Farris *et al.* 1995; Huelsenbeck *et al.* 1996). The most parsimonious trees were obtained by using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option, off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis & Huelsenbeck 1992). Characters were unweighted and unordered, branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein 1985). Trees were rooted to *Neofusicoccum ribis* and *Diplodia seriata,* which were treated as the outgroup taxa.

Baysian analysis was conducted on the same aligned combined dataset. First MrModeltest v2.2 (Nylander 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v3.1 (Ronquist & Huelsenbeck 2003) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. The Markov Chain Monte Carlo (MCMC) analysis of four chains started from random tree topology and lasted 10 000 000 generations, resulting in 10 000 000 saved trees. Burn-in was set at 500 000 generations after which the likelihood values were stationary, leaving 9950 trees from which the consensus trees and posterior probabilities were calculated. PAUP

4.0b10 was used to reconstruct the consensus tree and maximum posterior probability assigned to branches after a 50% majority rule consensus tree was constructed from the 9950 sampled trees.

Specific Primer Design and Validation

To design species-specific primers, the gene regions with the greatest sequence difference between *P. epicoccoides, P. eucalypti* and *P. destructans* were targeted. Only two gene regions, β-tubulin and elongation factor-1α, were sufficiently variable between *P. eucalypti* and *P. destructans* to allow for primer design.

Repeatability of the specific primers was tested by using 10 isolates of each *Phaeophleospora* spp. (*P. destructans*, CMW22553, 17919, 199832, 19936, 19844, 19886, 17918, 19906, 19909, 19910, *P. eucalypti*, CMW17912, 17915, 19916, 17917, MUCC432, 433, 434, 435, ,437, 438; *P. epicoccoides*, CMW5348, 22482, 22984, 22485, 22486, MUCC327, 424, 425, 426, 427). The isolates were amplified using specific βT and EF-1α primers (Table 2) and the same PCR conditions as Cortinas *et al.* (2006b). Thereafter, primers were tested for their specificity, primarily to closely related species, but also to five less related *Mycosphaerella* spp. (Table 3).

The ability of the primers to amplify DNA directly from fruiting bodies from infected leaves was determined. The samples were frozen in liquid nitrogen, ground and DNA extracted with CTAB as described previously (Wittzell 1999). DNA was then subjected to nested PCR, first using general βT and EF-1α primers and then the initial PCR product was diluted 1:5 and nested PCR conducted using the specific primers.

RESULTS

DNA sequence comparisons

Initially, 57 isolates representing 24 *Mycosphaerella* species and their anamorphs, including five species of *Phaeophleospora* found on *Eucalyptus* species and *P. eugeniae* the type species of the genus, were compared based on ITS sequence data (Table 1). The aligned data set consisted of 709 characters of which 127 bp were due to a large indel in two isolates of *P. epicoccoides* (MUCC327 and MUCC424) and this indel was excluded from the analyses. Of the remaining characters, 261 were parsimony informative. These data contained significant phylogenetic signal (P< 0.01; gl= - 0.41) to allow for meaningful analysis. Initial heuristic searches of unweighted characters in PAUP resulted in three most parsimonious trees of 910 steps (CI=0.56, RI=0.85). The *Phaeophleospora* species from *Eucalyptus*; *P. destructans, P. eucalypti, P. epicoccoides, P. toledana* and *M. ambiphylla* (which has a *Phaeophleospora* anamorph) grouped together in a strongly supported clade. This clade also included *M. nubilosa, M. cryptica, M. vespa, M. molleriana, Colleteogloeopsis zuluensis* and various undescribed '*Coniothyrium*' spp. (Figure 2). The ex-type culture of *P. destructans* (STEU1336 = CMW5219) was re-sequenced in this study and was distant from the isolate of *P. destructans* on GenBank (AF309614) (Crous *et al*. 2001). It was also distant from *P. eugeniae,* which is the type species of the genus, but close to *P. eucalypti* (Figure 2, TreeBASE SN2884). The ex-type culture of *P. eugeniae* (STEU1454 = CMW5351) was also re-sequenced and, while the new sequence was similar to that on GenBank (AF309613), it differed in the first 50bp of the ITS1 region. Based on results obtained for analysis of ITS sequence data, only species from the 'nubilosa clade' were retained for further study.

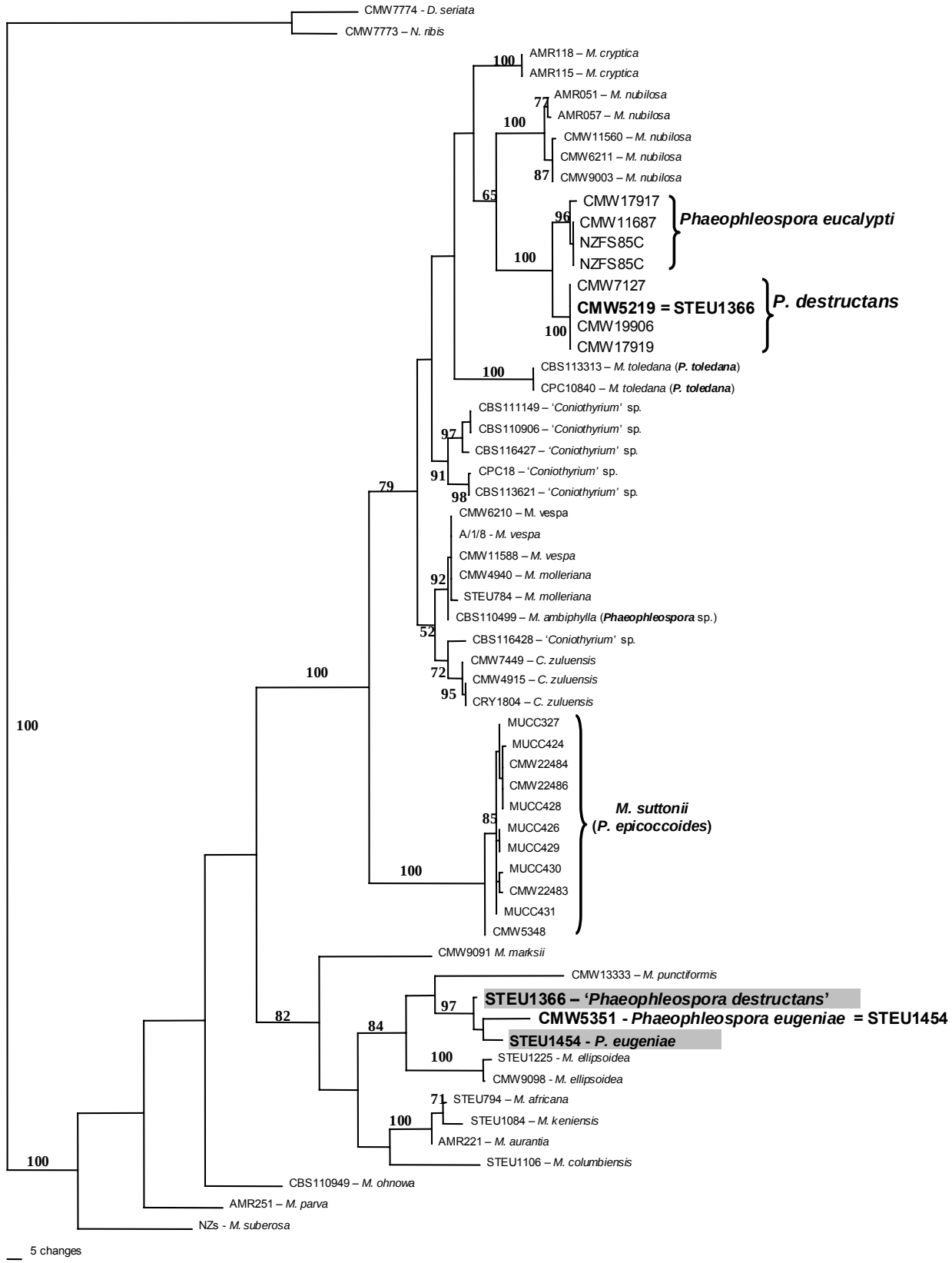


Figure 2. One of three most parsimonious phylogenetic trees of 977 steps obtained from analysis of ITS sequence data. Branch support (bootstrap values) is given above the branches. The sequences of the ex-type cultures of *P. eugeniae* and *P. destructans* from Crous *et al*. (2001) are shaded and those from the present study are in larger bold type. The tree is rooted to *Neofusicoccum ribis* and *Diplodia seriata.*

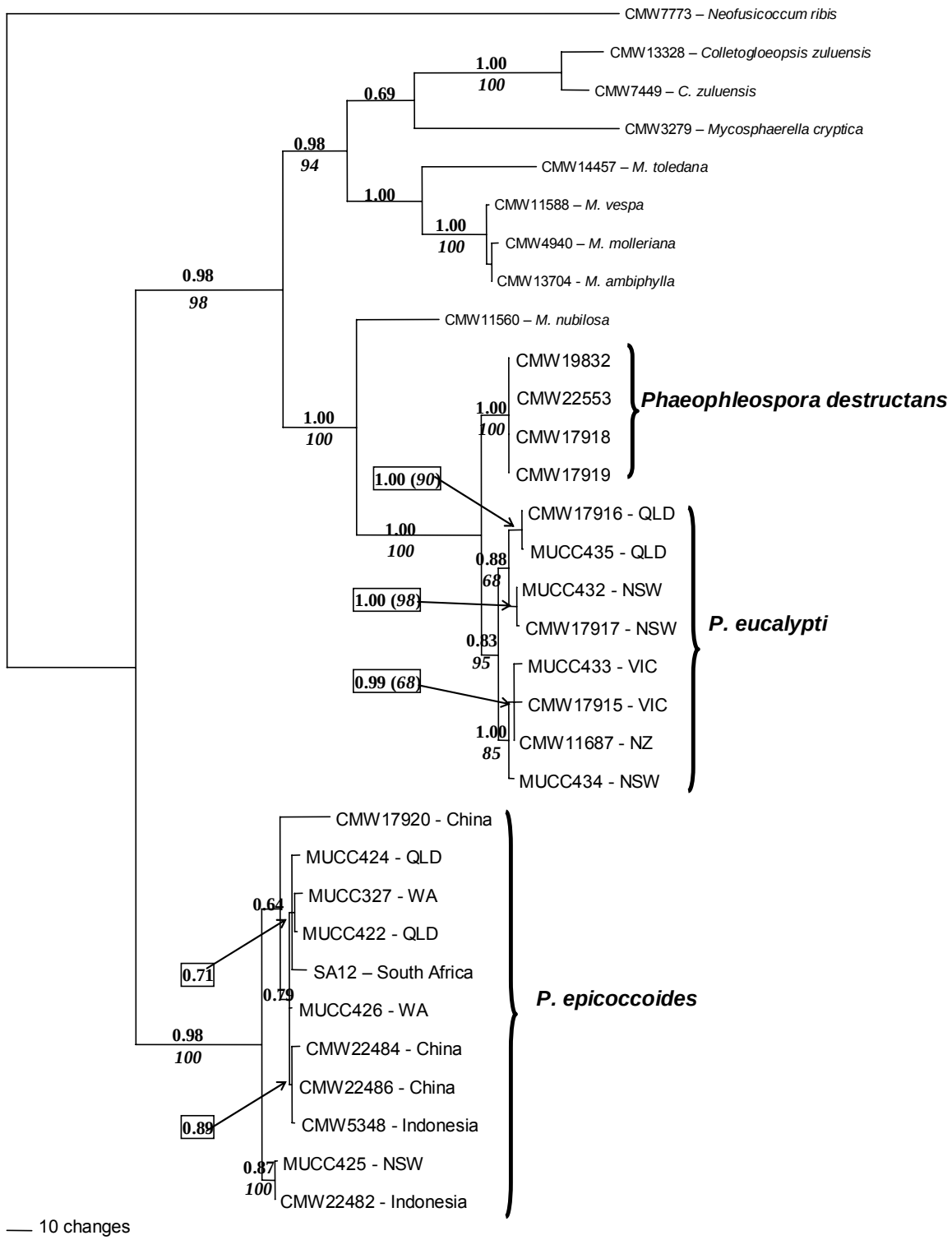


Figure 3. Consensus phylogram of 9950 trees resulting from Baysian analysis of the combined ITS-2, β-tubulin, EF-1α and CHS sequence data of *Phaeophleospora* isolates. Posterior probabilities of the node are indicated above the branches and bootstrap values from the parsimony analysis are indicated below branches in italics. Not all nodes with high posterior probabilities also have bootstrap support. The tree is rooted to *Neofusicoccum ribis.*

The multiple gene genealogies compared 31 isolates, including five *Phaeophleospora* species from *Eucalyptus.* The data set for the ATP-6 region could not be completed because of difficulties encountered in amplifying DNA for all isolates. The RPB2 region proved not to be informative and these two regions were excluded from the combined analysis. The aligned data set for the combined ITS, ß-tubulin, CHS and EF-1α sequences consisted of 1259 characters of which 352 were parsimony informative and were included in analysis. The partition homogeneity test showed significant (P = 0.001) difference between the data from the different gene regions (sum of lengths of original partition was 902, range for 1000 randomisations was 902-921). When the data sets were compared in pairs, the incongruence in the complete combined data set was actually due to incongruence between CHS and both the ITS and EF-1α datasets. On closer examination of the individual tree topography, the incongruence was due to the relationship of *M. cryptica* and *C. zuluensis* and not to the positions of the *Phaeophleospora* species (data not shown, sequence alignments are available from TreeBASE SN2884). Despite the fact that the partition homogeneity test showed a significant difference between data sets, they were nonetheless combined as suggested previously (Hognabba & Wedin 2003).

The combined data set contained significant (P<0.01, gl=-0.29). phylogenetic signal Heuristic search of unweighted characters in PAUP resulted in 18 most parsimonious trees of 937 steps (CI=0.68, RI=0.90). In the resultant tree (Figure 3, TreeBASE SN2884), *M. vespa, M. molleriana* and *M. ambiphylla* grouped together, while *P. destructans* and *P. eucalypti* were separated with 100% bootstrap support. The four isolates of *P. destructans* were identical and no polymorphisms were observed in any of the gene regions. There were 8 fixed polymorphic sites in the ITS region, 9 in the β-tubulin region and 24 in the EF-1α region separating *P. destructans* and *P. eucalypti*. The variable sites in the β-tubulin and EF-1α regions were used to design specific primers (Table 2). A table of polymorphic sites showing the variation between *P. destructans* and *P. eucalypti* isolates are presented in Table 4.

Phaeophleospora eucalypti isolates were further separated in three sub-groups, corresponding to isolates from Queensland, New South Wales and Southern New South Wales, Victoria and New Zealand (Figure 3). There were 18 polymorphic positions across the four gene regions among isolates

of *P. eucalypti* with two distinct profiles corresponding to geographic regions (Table 5). *Phaeophleospora epicoccoides* was the basal species of the group and has three strongly supported sub-groups (Figure 3). Although there were 26 polymorphic sites across the four gene regions, there was no geographic association linked to these polymorphisms (Table 6).

Table 3. Specific primers test results. Shaded cells indicate where the primers amplified non-specific DNA.

		P. destructans		***P. eucalypti***		***P. epicoccoides***	
Test species	**Code**	**ß-tubulin**	**EF1-α**	**ß-tubulin**	**EF1-α**	**ß-tubulin**	**EF1-α**
		198 bp	**204 bp**	**203 bp**	**229 bp**	**218 bp**	**173 bp**
Phaeophleospora destructans	CMW17919	**+**	**+**	+	-	-	-
P. eucalypti	CMW17916	-	-	**+**	**+**	-	-
P. epicoccoides	CMW5348	-	-	-	-	**+**	**+**
Mycosphaerella cryptica	CMW3279	-	-	-	-	-	-
M. vespa	CMW11588	-	-	-	-	-	-
M. toledana	CMW14457	-	-	+	-	-	-
Colletogloeopsis zuluensis	CMW7449	-	+(500bp)	-	-	-	-
M. nubilosa	CMW11560	-	-	-	-	-	-
M. molleriana	CMW4940	-	-	+	-	-	-
M. ambiphylla	CMW13704	-	-	+	-	-	-
P. eugeniae	CMW5351	-	+(400bp)	+		-	-
M. aurantia	MUCC258	-	-	-	-	-	-
M. marksii	MUCC214	-	Multiple bands	-	-	-	-
M. grandis	MUCC216	-	-	+	-	-	-
M. lateralis	MUCC436	-	-	+	-	-	-

Table 4. Polymorphic nucleotides from aligned sequence data of ITS, ß-tubulin and EF-1α gene regions showing the variation between *Phaeophleospora destructans* and *P. eucalypti* isolates.

Culture No.	Location	ITS2								ß-tubulin									EF-1α		
		73	127	202	215	229	231	233	234	136	198	218	222	223	224	236	267	277	110	251-273	276
										Phaeophleospora destructans											
CMW17919	China	C	C	T	T	C	C	A	A	G	C	C	C	G	G	T	C	A	T	+	C
CMW19832	Indonesia	C	C	T	T	C	C	A	A	G	C	C	C	G	G	T	C	A	T	+	C
CMW17918	Indonesia	C	C	T	T	C	C	A	A	G	C	C	C	G	G	T	C	A	T	+	C
CMW22533	Indonesia	C	C	T	T	C	C	A	A	G	C	C	C	G	G	T	C	A	T	+	C
										Phaeophleospora eucalypti											
CMW17916	Queensland	T	T	C	C	T	-	-	-	A	T	G	T	-	A	-	A	G	A	-	T
MUCC432	NSW	T	T	C	C	T	-	-	-	A	T	G	T	-	A	-	A	G	A	-	T
CMW17917	NSW	T	T	C	C	T	-	-	-	A	T	G	T	-	A	-	A	G	A	-	T
MUCC433	Victoria	T	T	C	C	T	-	-	-	A	T	G	T	-	A	-	A	G	A	-	T
CMW11687	NZ	T	T	C	C	T	-	-	-	A	T	G	T	-	A	-	A	G	A	-	T
MUCC434	NSW	T	T	C	C	T	-	-	-	A	T	G	T	-	A	-	A	G	A	-	T
MUCC435	Queensland	T	T	C	C	T	-	-	-	A	T	G	T	-	A	-	A	G	A	-	T
CMW17915	Victoria	T	T	C	C	T	-	-	-	A	T	G	T	-	A	-	A	G	A	-	T

Table 5. Positions of polymorphic nucleotides from aligned sequence data of ITS, ß-tubulin, EF-1α and CHS gene regions showing the variation between *Phaeophleospora eucalypti* isolates. Only parsimony informative nucleotides are shown. For comparison purposes polymorphisms not shared with the first isolate are highlighted.

Culture No.	Location	ITS2				ß-tubulin							EF-1α					CHS	
		104	153	220	221	108	127	129	134	242	250	280	33	36	71	182	226	197	242
CMW17916	Queensland	T	G	T	T	C	G	A	G	C	A	T	T	C	T	T	G	A	A
MUCC435	Queensland	T	G	T	T	C	G	A	G	C	A	T	T	C	T	T	G	G	A
MUCC432	NSW	C	T	C	T	T	C	-	A	C	A	C	T	C	T	T	G	A	A
CMW17917	NSW	C	T	C	T	T	C	-	A	C	A	C	T	C	T	T	G	A	A
MUCC434	NSW	C	T	T	C	T	G	A	A	T	G	C	C	C	C	C	G	G	G
MUCC433	Victoria	C	T	T	C	T	G	A	A	T	G	C	C	G	C	C	C	G	G
CMW11687	NZ	C	T	T	C	T	G	A	A	T	G	C	C	G	C	C	C	G	G
CMW17915	Victoria	C	T	T	C	T	G	A	A	T	G	C	C	G	C	C	C	A	G
AY626980	NZ	C	T	T	C														
AY626987	NZ	C	T	T	C														

Table 6. Positions of polymorphic nucleotides from aligned sequence data of ITS, ß-tubulin, EF-1α and CHS gene regions showing the variation between *Phaeophleospora epicoccoides* isolates. Only parsimony informative nucleotides are shown. For comparison purposes, polymorphisms not shared with the first isolate are highlighted.

		ITS2				ß-tubulin		EF-1α							CHS												
		130	174	215	232-234	344	348	62	191	210	213	215	273	288	88	112	121	130	161	179	185	188	194	200	220	228	232
CMW22482	Indonesia	T	T	T	+	C	C	T	T	C	C	C	A	-	A	T	C	A	-	A	G	G	C	G	A	G	G
CCT3	NSW	T	T	T	+	C	C	T	T	C	C	C	G	-	A	T	C	A	-	A	G	G	C	G	A	G	G
CMW17920	China	T	T	T	-	C	C	T	T	C	C	G	G	T	G	C	A	G	-	G	A	C	T	G	G	A	A
MUCC424	Queensland	C	T	C	+	G	A	T	A	T	C	C	G	-	G	C	A	G	C	G	A	C	T	-	G	A	A
MUCC422	Queensland	T	T	T	-	G	A	T	A	T	G	C	G	T	T	C	A	G	-	G	A	C	T	G	G	A	A
MUCC327	WA	T	T	C	+	G	A	T	A	T	G	G	G	T	T	C	A	G	-	G	A	C	T	G	G	A	A
SA12	South Africa	T	T	T	-	G	A	T	A	T	G	C	G	T	G	C	A	G	-	G	A	C	T	G	G	A	A
MUCC426	WA	T	G	T	+	G	A	T	T	T	G	C	G	T	G	C	A	G	-	G	A	C	T	G	G	A	A
CMW22484	China	C	T	C	+	G	A	C	T	T	G	C	G	T	G	C	A	G	-	G	A	C	T	G	G	A	A
CMW22486	China	T	T	T	+	G	A	C	T	T	G	C	A	-	G	C	A	G	C	G	A	C	T	-	G	A	A
CMW5348	Indonesia	T	T	T	+	G	A	C	T	T	G	C	G	T	G	C	A	G	-	G	A	C	T	G	G	A	A
CMW22485	China	C	T	C	+																						
MUCC428	Queensland	C	T	C	+																						
MUCC429	NSW	T	G	T	+																						
MUCC430	Queensland	T	T	T	+																						
MUCC431	NSW	T	T	T	+																						
CMW22483	Indonesia	T	T	T	+																						

Validation of species -specific primers

Gel photos showing reproducibility of the specific primers for *P. destructans, P. eucalypti* and *P. epicoccoides* are presented in Figure 4 (A-F).

Phaeophleospora destructans: DNA for ten isolates of *P. destructans* was amplified using the primers specific for β-tubulin and EF1-α. These primers were then tested on 10 closely related *Mycosphaerella* spp. and five less related species and none gave amplification products for the βt primers specific to *P. destructans* (data not shown). The EF1-α primer specific to *P. destructans* also amplified DNA of *C. zuluensis*, *P. eugeniae* and *M. marksii,* but the amplicons either contained multiple bands or were larger than the amplicon for *P. destructans* (Table 3). Both specific primer sets detected *P. destructans* directly from spores scraped from the surface of leaves. The βt primers specific for *P. destructans* also detected the presence of *P. eucalypti,* but the amplicon was larger than that obtained for *P. destructans* and it contained a double band.

Phaeophleospora eucalypti: DNA for all ten isolates of *P. eucalypti* was amplified using specific primers for ßt and EF1-α. None of *Mycosphaerella* spp. tested in this study gave amplification products for the EF1-α primers designed to be specific to *P. eucalypti* (Table 3). The ßt primers designed for *P. eucalypti* were not specific and amplified seven amplifying bands of the same size as those for *P. eucalypti* (Table 3). Only the EF1-α primers detected *P. eucalypti* from spores scraped from leaves.

Phaeophleospora epicoccoides: All ten isolates of *P. epicoccoides* gave amplification products using the ßt and EF1-α primers developed for this species. None of the *Mycosphaerella* spp. tested gave amplification products using these primers (Table 3). *In planta*, the EF1-α primer set detected the presence of *P. eucalypti* as well as *P. epicoccoides* and the ßt primer set detected presence of *P. epicoccoides* and *P. destructans* on leaf material.

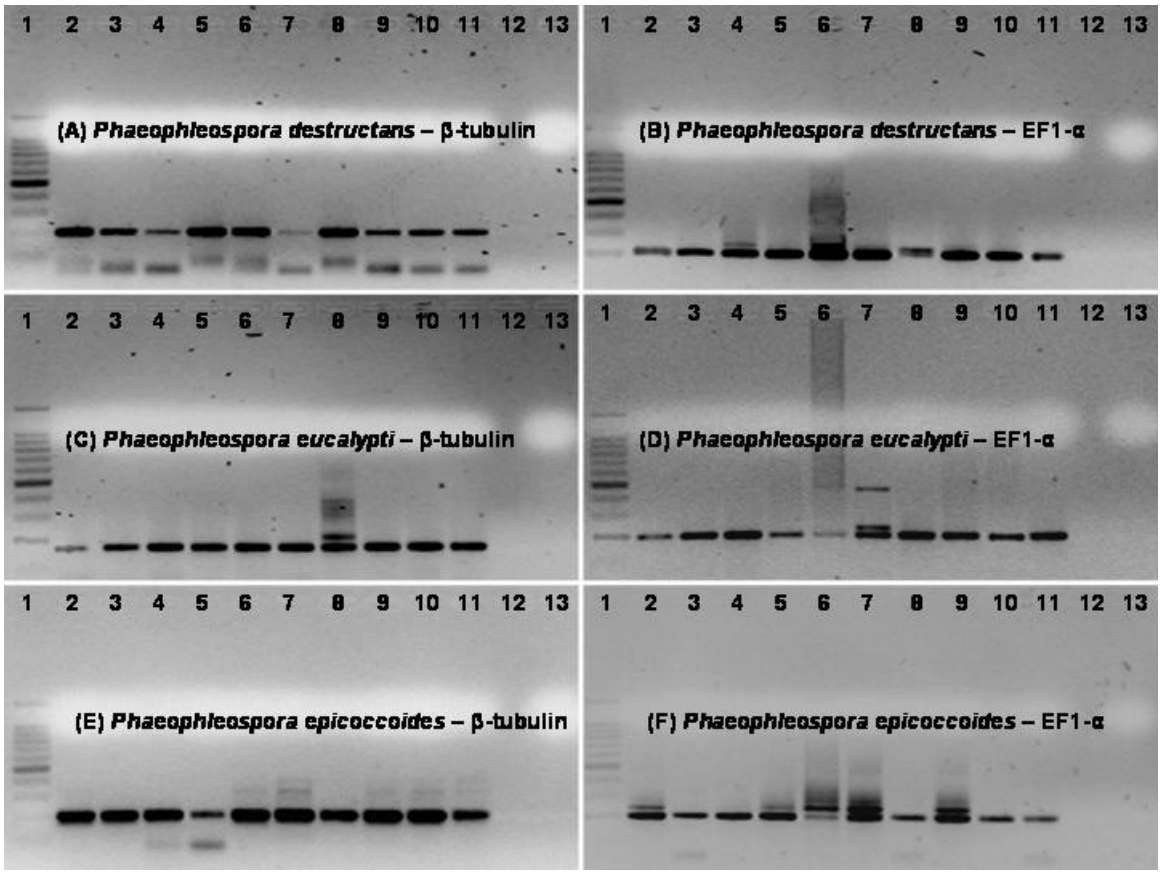


Figure 4 (A). Amplification products obtained following PCR with the β-tubulin primer set specific to *Phaeophleospora destructans* using DNA from 10 isolates of *P. destructans*. Lanes 1 to 13, 100 bp marker, CMW19886, CMW19909, CMW19936, CMW19910, CMW22553, CMW17919, CMW19906, CMW19844, CMW17918, CMW19832, empty lane, negative control.

Figure 4 (B). Amplification products obtained following PCR with the EF1 primer set specific to *Phaeophleospora destructans* using DNA from 10 isolates of *P. destructans*. Lanes 1 to 13, 100 bp marker, CMW19886, CMW19909, CMW19936, CMW19910, CMW22553, CMW17919, CMW19906, CMW19844, CMW17918, CMW19832, empty lane, negative control

Figure 4 (C). Amplification products obtained following PCR with the β-tubulin primer set specific to *Phaeophleospora eucalypti* using DNA from 10 isolates of *P. eucalypti*. Lanes 1 to 13, 100 bp marker, MUCC433, CMW17915, CMW17917, CMW17916, MUCC432, MUCC435, CMW17912, MUCC438, MUCC437, MUCC434, empty lane, negative control.

Figure 4 (D). Amplification products obtained following PCR with the EF1 primer set specific to *Phaeophleospora eucalypti* using DNA from 10 isolates of *P. eucalypti*. Lanes 1 to 13, 100 bp marker, MUCC433, CMW17915, CMW17917, CMW17916, MUCC432, MUCC435, CMW17912, MUCC438, MUCC437, MUCC434, empty lane, negative control.

Figure 4 (E). Amplification products obtained following PCR with the β-tubulin primer set specific to *Phaeophleospora epicoccoides* using DNA from 10 isolates of *P. epicoccoides*. Lanes 1 to 13, 100 bp marker, CMW22482, MUCC327, MUCC424, CMW5348, CMW22486, CMW22484, CMW22485, MUCC426, MUCC427, MUCC425, empty lane, negative control.

Figure 4 (F). Amplification products obtained following PCR with the EF1primer set specific to *Phaeophleospora epicoccoides* using DNA from 10 isolates of *P. epicoccoides*. Lanes 1 to 13, 100 bp marker, CMW22482, MUCC327, MUCC424, CMW5348, CMW22486, CMW22484, CMW22485, MUCC426, MUCC427, MUCC425, empty lane, negative control.

DISCUSSION

The current phylogenetic study has unequivocally shown *P. destructans* to be closely related to *P. eucalypti* and we have developed specific primers to easily distinguish between these two species. *Phaeophleospora destructans* is unknown in Australia and is considered a major biosecurity threat. However, based on symptoms it is hard to distinguish between *P. eucalypti*, which is well-known in Australia and *P. destructans*. Thus, the specific primers will be very useful for detection and surveillance activities.

In a former study, *Phaeophleospora* species emerged in two separate clades (Crous *et al.* 2001). One of these clades included *P. eucalypti* and *P. epicoccoides* and the other accommodated *P. eugeniae* and *P. destructans* (Crous *et al.* 2001). All the isolates of *P. destructans* examined in the present study, including the ex-type culture (STE-U1366 = CMW5219), had identical ITS sequence data, which were different to the single sequence previously lodged in GenBank (isolate STE-U 1366, AF309613). Consequently, all *Phaeophleospora* species from *Eucalyptus* species cluster together and they are closely related to the important *Eucalyptus* pathogens, *Colletogloeopsis zuluensis, M. cryptica* and *M. nubilosa.* In contrast, these fungi are distantly related to *P. eugeniae*.

The sequence data obtained in this study for four isolates of *P. destructans,* three from Indonesia and one from China, were identical for all six gene regions examined. This finding is unusual as some variability is usually observed in sequence data between isolates of the same species, especially when more than one region of origin is considered. The limited variability among isolates of *P. destructans* supports the hypothesis of selection pressure resulting in the adaptation of a limited number of genotypes to a new host (*Eucalyptus* in Sumatra, Indonesia) followed by dispersal of these genotypes throughout Asia. In the present study, we found no informative characters in the RPB2 and CHS regions that could separate *P. destructans* from *P. eucalypti*. There were, however, a few stable differences between the two species in the sequences for the ITS2 and β-tubulin regions. The most variable gene region was EF1-α where a 22 bp indel separated these species. For ITS2, β-tubulin and CHS gene regions there were more polymorphic sites among isolates of *P. eucalypti* than between *P. destructans* and *P. eucalypti*. This suggests that while *P. destructans* emerged as a major *Eucalyptus*

pathogen in Asia, it may have very recently evolved from *P. eucalypti,* to which it is very closely related. Where this adaptation could have occurred, however, remains a mystery as *P. eucalypti* has not been detected in Asia.

The sequence data for different *P. eucalypti* isolates were variable and analysis resulted in the isolates residing in different sub-groups based on their origin. As isolates from New Zealand grouped with isolates from Victoria and southern New South Wales, *P. eucalypti* might have been moved to New Zealand from this region. Phylogeographic studies are required to test this hypothesis appropriately (Carbone & Kohn 2001; Kasuga *et al.* 2003).

Many polymorphic sites were observed amongst the sequence data sets for isolates of *P. epicoccoides*, but the groupings did not reflect any obvious pattern relating to origin or other characteristics of the isolates. Unlike *P. eucalypti*, this species is widely distributed throughout most *Eucalyptus* growing regions of the world. The lack of phylogenetic grouping amongst isolates with variable sequence data, probably refects anthropogenic movement of germplasm and multiple introductions of the fungus into new areas. *Phaeophleospora epicoccoides* is known to be a morphologically variable species and it may represent a species complex rather than a single taxon (Crous & Wingfield 1997a). Population genetic studies and large numbers of isolates from different locations, especially in Australia are required to resolve this question.

Efforts to develop species specific primers for *P. destructans, P. eucalypti* and *P. epicoccoides* reflected the close relatedness between these species and the variably within the species. Nonetheless a suite of species specific primers have been developed that allow for simple distinction between these species. Primers based on the EF1-α region distinguished between all three species and primers for the β-tubulin regions provided reliable detection of *P. destructans* and *P. epicoccoides.* Specific primers based on EF1-α sequences were able to detect *P. eucalypti* and *P. destructans* directly from plant samples. The β-tubulin primers developed to detect *P. epicoccoides* also showed a faint positive band for *P. destructans*, while EF1-α primers developed to detect *P. epicoccoides* showed a faint band for *P. eucalypti* from leaf material. While this result may be considered confusing, we believe this reflects

duel infection as *P. epicoccoides* is very often present on the same lesion together with *P. eucalypti* and *P. destructans* (Burgess *et al.* 2006a).

Phaeophleospora destructans is a devastating pathogen of *Eucalyptus* as yet undetected in Australia. Since the fungus has been detected in East Timor, which is very close to the Australian border, it is a potential threat to the biosecurity and biodiversity of Australia's vast native *Eucalyptus* forests. Its early detection in Australia is important and the Australian Quarantine and Inspection Service (AQIS) regularly inspects *Eucalyptus* species in Australia and neighbouring countries for pathogens including *P. destructans*. Because the symptoms caused by *P. destructans* can be almost identical to those associated with *P. eucalypti* and *P. epicoccoides*, unequivocal identification procedures are important. The DNA sequence data for many gene regions and the specific markers produced in this study should assist in this process.

CHAPTER 3

Phylogenetic reassessment supports accommodation of *Phaeophleospora* and *Colletogloeopsis* from eucalypts in *Kirramyces*

Published as: Andjic V, Barber PA, Carnegie AJ, Hardy GEStJ, Wingfield MJ, Burgess TI, 2007. Phylogenetic reassessment supports accommodation of *Phaeophleospora* and *Colletogloeopsis* from eucalypts in *Kirramyces*. *Mycological Research* **111**(10): 1184-1198.

Minor authors' contributions: Burgess TI and Hardy GEStJ were supervisors, Carnegie AJ provided the leaf material and advice on species description, Barber PA and Wingfield MJ provided guidance and expertise in taxonomy.

ABSTRACT

Species of *Phaeophleospora* are anamorphs of *Mycosphaerella* and they include some of the most serious foliar pathogens of *Eucalyptus* spp. grown in plantations world-wide. Pathogens assigned to this genus and occurring on *Eucalyptus* spp. were previously treated in *Kirramyces* and they are also phylogenetically closely related to other anamorphs of *Mycosphaerella* residing in the genus *Colletogloeopsis*. The primary aim of this study was to consider the appropriate taxonomic placement of these species. To achieve this goal, morphological characteristics and DNA sequence data from the ITS and translation EF1-α gene regions were used to compare the type species *P. eugeniae*, *Phaeophleospora* spp. and *Colletogloeopsis* spp. occurring on eucalypts, using ex-type cultures and herbarium specimens. Phylogenetic data and morphological comparisons supported the separation of *P. eugeniae* from *Phaeophleospora* species occurring on eucalypts. The name *Phaeophleospora* is retained for *P. eugeniae* and the name *Kirramyces* is resurrected for the species occurring on eucalypts (genera *Eucalyptus*, *Corymbia* and *Angophora*). Sequence data from the type specimens of two previously described species of *Kirramyces*, *K. lilianiae* and *K. delegatensis*, show they reside in a clade with other *Kirramyces* spp. Morphological and DNA sequence comparisons also showed that there is considerable overlap between species of *Phaeophleospora* and *Colletogloeopsis* from eucalypts. Based on these findings, *Colletogloeopsis* is reduced to synonymy with the older *Kirramyces* and the description of *Kirramyces* is emended to include species with aseptate as well as multiseptate conidia produced in acervuli or pycnidia. Two new species of *Kirramyces*, *K. angophorae* and *K. corymbiae*, are also described.

INTRODUCTION

Phaeophleospora spp. are anamorphs of *Mycosphaerella* that cause leaf and shoot blight diseases on many plants including members of the families *Myrtaceae*, *Proteaceae*, *Malvaceae*, *Elaeocarpaceae*, and *Sapotaceae*. The genus *Phaeophleospora* was introduced to accommodate the dark form of *Phleospora* (*Phloeospora* Wallr.) by Rangel (1916). However, most taxonomists regarded the monotypic genus *Phaeophleospora* Rangel as a *nomen dubium* because the mode of conidiogenesis and the form of the conidia were not documented in the type description (Sutton 1977). Up until 1997, *Phaeophloespora* included only two species; *P. eugeniae* Rangel occurring on *Eugenia uniflora* L. and *P. elaeocarpi* Bond occurring on *Elaeocarpus* spp. (Bond 1947).

Confusion regarding the taxonomic placement of *Phaeophleospora* first emerged when a suitable name was sought for a *Pseudocercospora* spp. causing widespread damage on *Eucalyptus* in South Africa. Type specimens of *Cercospora epicoccoides* Cooke & Massee and *C. eucalypti* Cooke & Massee were examined and it was concluded that neither of these species were correctly placed in the genus *Cercospora* Fres. (Crous *et al.* 1989). Thus, *C. epicoccoides* was shown to reside in *Phaeoseptoria* Speg. where it was known as *Phaeoseptoria eucalypti* Hansf. Walker, Sutton & Pascoe (1992) later compared *Ph. eucalypti* with the type specimen of the genus *Phaeoseptoria*, *Ph. papayae* Speg. and noted that *Ph. eucalypti* differed from the type specimen in having large, brown, cylindrical, rough-walled, percurrently proliferating conidiogenous cells, that were not present in the type species. *Phaeoseptoria papayae* had also previously been redescribed by Morgan-Jones (1974) and both he and Walker *et al.* (1992) found large, brown, cylindrical, rough-walled, percurrently proliferating conidiogenous cells were not present.

In a search for a suitable genus for *Ph. eucalypti*, several genera were considered (Walker *et al.* 1992). These included *Scoleciasis* Roum & Fautrey, *Sonderhenia* Swart & Walker and *Stagonospora* Sacc., but none were suitable because they were all characterised by smooth-walled conidiogenous cells and distoseptate conidia (*Sonderhenia*) or smooth-walled, hyaline conidia (*Stagonospora*). *Phaeoseptoria eucalypti* was therefore removed from *Phaeoseptoria* and the new genus *Kirramyces* J. Walker, B. Sutton & Pascoe was introduced for species with pycnidial conidiomata, brown, euseptate, cylindrical

to narrowly obclavate rough-walled conidia and brown roughened annellidic conidiogenous cells (Walker *et al.* 1992). The genus included three taxa, *K. epicoccoides* (Cooke & Massee) J. Walker, B. Sutton & Pascoe and *K. lilianiae* J. Walker, B. Sutton & Pascoe, characterised by brown rough-walled conidia and *K. eucalypti* (Cooke & Massee) J. Walker, B. Sutton & Pascoe with pale-yellowish-brown, finely roughened conidia. Walker *et al.* (1992) recognized the similarity in conidial size and shape between *K. eucalypti* and the *Stagonospora delegatensis* R.F. Park & Keane anamorph of *Mycosphaerella delegatensis* R.F. Park & Keane but they noted that *S. delegatensis* differed from *Kirramyces* spp. in having paler, slightly less tapered and smooth conidia. These features also indicated that it was poorly accommodated in *Stagonospora*, but due to the lack of a suitable number of collections, the taxonomic position of this fungus was not resolved. Sankaran *et al*. (1995) later reduced *S. delegatensis* to synonymy with *K. eucalypti* without providing an explanation for their decision.

Crous *et al*. (1997a) redescribed *Phaeophleospora eugeniae* based on the collection and designation of a neotype. In their study, it was concluded that *P. eugeniae* resembled species residing in *Kirramyces,* and *Kirramyces* was reduced to synonymy under the older name *Phaeophleospora*. Differences were noted between *Phaeophloespora* and *Kirramyces*, particularly in the gradient of pigmentation and number of septa in the conidia, but they did not consider these sufficiently important to justify separation at the generic level Crous *et al.* (1997a). Other species of *Kirramyces* that had previously been described by Walker *et al*. (1992) as well as *K. proteae* B. Sutton, *K. hebes* W.P. Wu, B. Sutton & Gange, *K. phormii* Naito and *K. destructans* M.J. Wingf. & Crous, were re-allocated to *Phaeophleospora* (Crous *et al.* 1997a).

Maxwell *et al.* (2003) selected *Phaeophloespora* as the anamorph of *M. ambiphylla* A. Maxwell because the conidia of the fungus were produced in pycnidia. These authors, however, noted that aseptate conidia had not previously been described for species residing in *Phaeophleospora*. Amongst *Mycosphaerella* anamorphs, aseptate conidia are more typical of *Colletogloeopsis* spp. with acervular conidiomata, however, the anamorph of *M. ambiphylla* produced conidia in pycnidia rather than acervuli. More recently, Crous *et al.* (2004) described the anamorph of *M. toledana* Crous & Bills in

the genus *Phaeophleospora* because the conidiomata were pycnidial rather than acervular. This species like the anamorph of *M. ambiphylla,* produces aseptate conidia.

In a taxonomic re-evaluation of *Coniothyrium zuluensis* M.J. Wingf., Crous, T.A. Cout., Cortinas *et al.* (2006b) showed that this pathogen which produces conidia in pycnidia, always clusters in the same clade as *Colletogloeopsis nubilosum* Ganapathi & Corbin and *Colletogloeopsis molleriana* Crous & M.J. Wingfield whose conidia are produced in acervuli. Based on phylogenetic data, Cortinas *et al.* (2006b) emended the description of *Colletogloeopsis* to accommodate *Coniothyrium*-like anamorphs residing in *Mycosphaerella*, and included pycnidial as well as acervular conidiomata in this description. The authors could not place this fungus in the genus *Phaeophleospora*, as the type species *P. eugeniae* was found to be phylogenetically distant from *C. zuluensis* (Cortinas *et al.* 2006b). Likewise, in Chapter 2, the phylogenetic relationship of *Phaeophleospora* species from eucalypts, was investigated, and these species were found to be phylogenetically distant from the ex-type culture of *P. eugeniae* but close to *C. zuluensis* and *M. nubilosa* (Cooke) Hansf. As a result, *Phaeophleospora* was no longer suitable to accommodate *Colletogloeopsis*-like species with pycnidial conidiomata as had previously been true for the anamorphs of *M. ambiphylla* (Maxwell *et al.* 2003) and *M. toledana* (Crous *et al.* 2004).

Species of *Phaeophloeospora sensu lato* and *Colletogloeopsis* are common and important pathogens of eucalypts. New species that might reside in either of these genera are being collected regularly. For practical taxonomic reasons and for the establishment of appropriate quarantine regulations, these fungi require appropriate names. The present emerged from the collection of apparently new species in these two genera, and the assignment of these new species to the appropriate genus necessitated a detailed phylogenetic and morphological study of related fungi. These included type specimens representing *Phaeophleospora, Kirramyces* and *Colletogloeopsis*. The correct taxonomic placement of these genera was thus reassessed; suitable synonymies and combinations are proposed in *Kirramyces* and new species are described. *Phaeophleospora* remains as the monotypic genus for *P. eugeniae*.

MATERIALS AND METHODS

Isolates

Fungal isolation and culture maintenance were performed as in Chapter 2. Herbarium specimens were obtained for *Phaeophleospora lilianiae* (DAR 3833) and *P. delegatensis* (DAR 45718b), species for which an ex-type culture or sequence data do not exist. Herbarium material used for measurements included: *P. destructans* (PREM 59259, PREM 59261), *P. epicoccoides* (PREM 59258, PREM 59260, MURU 422, MURU 423) and *P. eucalypti* (MURU 424, MURU 425). The cultures used in this study are maintained in the culture collections of Murdoch University (MUCC), the Forestry and Agricultural Biotechnology Institute, University of Pretoria (CMW), New South Wales, Plant Pathology Herbarium (DAR), and State Forests of New South Wales (NSWF), Australia. Herbarium specimens of new collections have been lodged at DAR and NSWF. Herbarium abbreviations used are those given in Index Herbariorum (Holmgren & Holmgren 1998).

DNA extraction and PCR from cultures

Isolates were grown on 2% MEA at 20°C for 4 weeks and the mycelium harvested and placed in 1.5 ml sterile Eppendorf® tubes. Harvested mycelium was frozen in liquid nitrogen, ground to a fine powder and genomic DNA extracted using a hexadecyl trimethyl ammonium bromide (CTAB) modified protocol of Graham *et al.* (1994). Six hundred µl of extraction buffer (2% CTAB; 100 mM Tris-HCl pH8.0, 1.4M NaCl 2% PVP-40 100 µg/ml Proteinase K, 100 µg/ml RNAse A) was added per 60 mg of freeze-dried mycelium and incubated at 55°C for 20 min. After incubation, the tubes were centrifuged for 2 min at 11 000 x g, and the supernatant transferred to a new tube and extracted with equal volume of chloroform isoamyl alcohol 24:1 (IAC), centrifuged for 20 sec at 11 000 x g, the upper aqueous phase transferred to a new tube and 0.1 volumes of 7.5M ammonium acetate and 2 volumes of 100% added to precipitate the DNA. The tubes were inverted a few times, incubated at 20°C for 60 min, centrifuged for 1 min at 11 000 x g, the supernatant discarded and the DNA pellet washed with 1 ml of 70% ice-cold ethanol and centrifuged at 1000 x g for 1 min. The ethanol was decanted and the DNA allowed to air dry for 15 min. The DNA was resuspended in 30 µl Ultrapure

PCR grade water. ITS1, ITS2 and 5.8S regions of the ribosomal DNA operon was amplified using primers ITS-1F (5' CTT GGT CAT TTA GAG GAA GTA A 3') (Gardes & Bruns 1993), ITS-4 (5'TCC TCC GCT TAT TGA TAT GC 3') (White *et al.* 1990), and part of the translation elongation factor 1-α region was amplified using primers EF1-728F (5' CAT CGA GAA GTT CGA GAA GG 3') and EF1-986R (5' TAC TTG AAG GAA CCC TTA CC 3') (Carbone & Kohn 1999).

PCR was performed using GeneAmp PCR System 2700 Thermal Sequencer (Applied Biosystems Australia). Each 25 µl reaction mixture contained 1 x PCR Polymerisation buffer (67 mM Tris-HCl, 16.6 mM Ammonium sulphate, 0.45% Triton X-100, 0.2 mg/mL, Gelatine 0.2 mM of each dNTP's) (Fisher Biotech, Australia), 25 mM $MgCl_2$ (Fisher Biotech, Australia), 0.6 pmol of each primer (GeneWorks, Australia), approximately 5 ng of DNA and 1 unit of Taq DNA Polymerase (Fisher Biotech, Australia). During the PCR reaction, the DNA first was denatured at 94° C for 2 min, followed by 35 cycles of denaturation (94° C for 30 sec), annealing (55° C for 45 sec) and elongation (72° C for 1 min) and ended with a final elongation step at 72° C for 5 min. To detect possible contamination in the amplification reaction, a negative control that contained all reaction components except the fungal template DNA, was used with every reaction. The PCR products were visualised on 1% agarose gel containing ethidium bromide using an UV transluminator and purified with Ultrabind®DNA purification kit (MO BIO Laboratories) following the manufacturer's instructions.

DNA extraction and amplification from herbarium specimens

In the case where ex-type cultures were not available for species required to define genera, DNA extractions were made directly from herbarium specimens representing the types. Several individual pycnidia or acervuli were carefully removed from the herbarium specimens and transferred to a 1.5 ml tube and ground with liquid nitrogen to a fine powder. DNA was extracted with CTAB extraction buffer as described previously (Wittzell 1999).

Initially the DNA from the herbarium specimens was amplified using the internal transcribed spacer primers ITS-1F and ITS-4, followed by nested PCR using the primers ITS-1 (5' GTA TCG ATG AAG AAC GCA GC 3') and ITS2 (GCTCGGTTCTTCATCGATGC) (White *et al.* 1990) primers with 1:5

dilution of initial PCR product as template. PCR mixture and running conditions were as described above.

Some isolates showed a false positive amplification when subjected to nested PCR, thus direct PCR was used and the running conditions were changed. The Mg concentration was increased to 4 mM and 0.8 % bovine serum albumin was added to each reaction. The DNA was denatured at 94° C for 7 min, followed by 40 cycles of denaturation (94° C for 2 min), annealing (45° C for 1 min) and elongation (72° C for 2 min) and ended with a final elongation step at 72° C for 10 min.

Phylogenetic analyses

In order to compare species of *Phaeophleospora,* sequences in addition to those derived in this study were obtained from GenBank (Table 1). Sequence data were assembled using Sequence Navigator version 1.01 (Perkin Elmer, Melbourne, Australia) and aligned in Clustal X (Thompson *et al.* 1997). Manual adjustments were made visually by inserting gaps where necessary. All sequences derived in this study were deposited in GenBank and accession numbers are shown in Table 1.

Analyses were performed on the combined dataset of complete ITS and EF-1α sequences, after a partition homogeneity test (PHT) had been performed in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b 10 (Swofford 2003) to determine whether sequence data from the two gene regions were statistically congruent (Farris *et al.* 1995; Huelsenbeck *et al.* 1996). Parsimony analysis with heuristic search was performed using (PAUP) version 4.0b10 (Swofford 2003) with random stepwise addition in 100 replicates with the tree bisection-reconnection branch-swapping option and the steepest-descent option off. All ambiguous and parsimony-uninformative characters were excluded; gaps were treated as a fifth character. MaxTrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees saved. Estimated levels of homoplasy and phylogenetic signal; tree length (TL), consistency index (CI) and retention index (RI) were determined (Hillis & Huelsenbeck 1992). Characters were unweighted and unordered branch and branch node support was determined using 1000 bootstrap replicates with equal probability (Felsenstein 1985). Trees were rooted to *Neofusicoccum ribis*, which was treated as the outgroup taxon.

Bayesian analysis was conducted on the same aligned and combined dataset as that used in the distance analysis. First, MrModeltest v2.2 (Nylander 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v3.1 (Ronquist & Huelsenbeck 2003) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Two independent runs of Markov Chain Monte Carlo (MCMC) using 4 chains were run over 10 000 000 generations. Trees were saved each 10 000 generations, resulting in 10 001 trees. Burn-in was set at 500 001 generations (*i.e*. 51 trees), well after the likelihood values converged to stationary, leaving 9950 trees from which the consensus trees and posterior probabilities were calculated.

Morphological comparisons

In order to re-assess the taxonomical status of *Phaeophleospora* spp. associated with eucalypts and to determine the correct generic placement for apparently undescribed species obtained from *Corymbia maculata* and *Angophora floribunda*, representative isolates of the unknown species and representative isolates of *P*. *destructans*, *P*. *epicoccoides* and *P*. *eucalypti* were compared *in vivo* and *in vitro* with type specimens and with previous observations from published literature. The only available isolate of *P*. *eugeniae* (CMW5351) was sterile in culture and it could not be used for morphological comparisons. Ex-holotype cultures of *P*. *elaeocarpi*, *P*. *delegatensis* and *P*. *lilianiae* do not exist. The herbarium specimens were examined for *P*. *epicoccoides* (DAR 6338), *P. eucalypti* (*Septoria normae*) (DAR 65274), *Phaeophleospora lilianiae* (DAR 3832, DAR 3833), *P. delegatensis* (DAR 45718b), *P. eugeniae* (IMI 372655), *Colletogloeopsis nubilosum* (PDD 37677) and *C. molleriana* Thum. (PREM 54395). Where morphological characteristics of species could not be determined from culture or herbarium specimens, data from published literature were included. Available isolates were characterised using cultural characteristics useful for *Phaeophleospora* species separation, such as conidial pigmentation, number of septa and conidial size (Crous *et al.* 1997a).

Four replicates of each isolate used in comparisons were prepared using 55 mm diameter Petri plates and 2% Malt Extract Agar (MEA). After 30 days, cultures were photographed and squash mounts of fruiting structures were prepared on slides in lactoglycerol and observed under an Olympus BH2 light

microscope. Each isolate was assessed for conidial size, shape, pigmentation and number of septa. Unknown species were also assessed for growth rate after one month growing at 20° C in the dark. The growth rate was determined by measuring perpendicular colony diameters. Wherever possible, 30 measurements of all taxonomically relevant structures were recorded for each species and the extremes have been presented in parentheses. Colony colour for the unknown species was described using notations in the Munsell®Soil Color Charts (Gretag Macbeth, New Windsor, New York, revised 2000). Measurements of conidial size were obtained using the image analysis software Olysia BioReport 3.2 soft imaging system. Data analyses were performed using descriptive statistics in Microsoft Excel. The drawings were prepared using a drawing tube attached to a BH2 Olympus Microscope. These drawings were then scanned on a flatbed scanner at 300 dpi, imported into the software program Macromedia Freehand version 10, and traced into a vector file. This file was then imported into Adobe Photoshop version, airbrushed and stippled using the Andromeda Series 3 Screen Filter (Barber, accepted in Fungal Diversity).

Table 1. Isolates considered in the phylogenetic study.

Culture no [1]	teleomorph	anamorph	Host	Location	Isolator	GenBank ITS	GenBank EF-1α
CMW 7127		*P. destructans*	*E. grandis*	Sumatra, Indonesia	M.J. Wingfield	DQ632698	EF011658
CMW 17919		*P. destructans*	*E. urophylla*	China	T.I. Burgess	DQ632701	DQ632729
CMW 17917		*P. eucalypti*	*E. grandis x E. tereticornis*	NSW, Australia	A.J. Carnegie	DQ632711	DQ632725
CBS 113992, CMW 11687		*P. eucalypti*	*E. nitens*	New Zealand	M. Dick	DQ240001	DQ235115
CMW 22484	*M. suttonii*	*P. epicoccoides*	*Eucalyptus* sp.	China	T.I. Burgess	DQ632705	DQ632714
MUCC 426	*M. suttonii*	*P. epicoccoides*	*E. globulus*	WA, Australia	S. Jackson	DQ632704	DQ632715
CMW 5348, STE-U 1346	*M. suttonii*	*P. epicoccoides*	*Eucalyptus* sp.	Indonesia	M.J. Wingfield	AF309621	DQ240170
CBS 113313, CMW 14457	*M. toledana*	*P. toledana*	*E. globulus*	Spain	P.W. Crous	AY725581	DQ235120
CMW 5351		*P. eugeniae*	*Eugenia uniflora*	Brazil	M.J. Wingfield	DQ632710	EF011663
CMW 11560	*M. nubilosa*		*E. globulus*	Tasmania	A. Milgate	DQ658232	DQ240176
CBS 114708, CMW 9003	*M. nubilosa*		*E. nitens*	South Africa	G. C. Hunter	AF449099	DQ235112
CBS 110975, CMW 3279	*M. cryptica*	*C. nubilosum*	*E. globulus*	Australia	A.J. Carnegie	AY309623	DQ235119
CBS 117262, CMW 7449		*C. zuluensis*	*E. grandis*	South Africa	L. Van Zyl	DQ240021	DQ240155
CBS 113399, CMW 13328		*C. zuluensis*	*E. grandis*	South Africa	L. Van Zyl	DQ240018	DQ240172
CBS 110499, CMW 14180	*M. molleriana*	*C. molleriana*.	*E. globulus*	Western Australia	A. Maxwell	AY150675	DQ240169
CBS 111164, CMW 4940	*M. molleriana*	*C. molleriana*	*Eucalyptus* sp.	Portugal	S. McCrae	AF309620	DQ235104
CBS 117924, CMW 11588	*M. molleriana*	*C. molleriana*	*E. globulus*	Tasmania		DQ239968	DQ240167
CBS 116154, CMW 4945	*M. africana*		*Eucalyptus*	South Africa	P.W. Crous	AF309602	DQ235099
CBS 116155, CMW 3026	*M. africana*		*Eucalyptus*	South Africa	P.W. Crous	DQ267577	DQ235098
CBS 111011, CMW 5147	*M. keniensis*		*Eucalyptus*	Kenya	T.A Couthino	DQ246259	DQ235100
CMW 4934	*M. ellipsoidea*	*Uwebraunia ellipsoidea*	*Eucalyptus*	South Africa	M.J. Wingfield	DQ246253	DQ235129
CMW 5166	*M. ellipsoidea*	*U. ellipsoidea*	*Eucalyptus*	South Africa	M.J. Wingfield	DQ246254	DQ235127
CBS 110969, CMW 4944	*M. colombiensis*		*E. urophylla*	Colombia	M.J. Wingfield	DQ204744	DQ211660
CBS 110967, CMW 11255	*M. colombiensis*		*E. urophylla*	Colombia	M.J. Wingfield	DQ204745	DQ211661
CMW 7773	*Neofusicoccum ribis*		*Ribes sp.*	New York, USA	B. Slippers	AY236936	AY236878
CBS 120495, DAR 77445		***Kirramyces corymbiae***	***Corymbia maculata***	**NSW, Australia**	**A.J.Carnegie**	EF011657	EF011661
CBS 120496, DAR 77446		***K. corymbiae***	***Corymbia maculata***	**NSW, Australia**	**A.J.Carnegie**	EF011656	EF011662
CBS 120493, DAR 77452		***K. angophorae***	***Angophora floribunda***	**NSW, Australia**	**A.J.Carnegie**	EF011653	EF011660
CBS 120494, DAR 77451		***K. angophorae***	***Angophora floribunda***	**NSW, Australia**	**A.J.Carnegie**	EF011652	EF011659

[1]Designation of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht Netherlands; CMW = Tree Pathology Co-operative Program Forestry and Agricultural Biotechnology Institute University of Pretoria South Africa; STE-U = Stellenboch University South Africa; MUCC = Murdoch University culture collection, Australia; DAR = New South Wales, Plant Pathology Herbarium, Australia.

RESULTS

Phylogenetic analyses

After repeated attempts to amplify the whole ITS region of herbarium specimens of *P. delegatensis* and *P. lilianiae* sequence data for the ITS 1 region were obtained. For both species, BLASTn searches on GenBank (http://www.ncbi.nlm.nih.gov/) returned the closest match as *M. molleriana*. A phylogenetic analysis based solely on ITS1 region placed *P. delegatensis* (EF011654) and *P. lilianiae* (EF011655), in a strongly supported clade with all other *Phaeophleospora* spp. and *Colletogloeopsis* spp. from eucalypts, including the newly described species *C. stellenboschiana* (CBS116428) and *C. gauchensis* (CMW17328, CMW17330) and undescribed *Colletogloeopsis* spp. (CBS111149, CBS110906, CBS116427, CPC18, CBS113621), and far from the type species of *Phaeophleospora, P. eugeniae* (data not shown, TreeBASE SN3058).

The multiple gene genealogies for ITS and EF-1α sequence data compared 28 isolates representing *Phaeophleospora* spp. (including *P. eugeniae*) and *Colletogloeopsis* spp. from *Eucalyptus* and two unknown taxa isolated in this study from *Corymbia* and *Angophora*. The aligned data set for the combined gene regions consisted of 967 characters of which 428 were parsimony informative and used in the analyses. The partition homogeneity test showed significant (P=0.001) difference between the data from different gene regions (sum of lengths of original partition was 1406 range for 1000 randomisations was 1409-1428). However, the differences in the topology between the trees was not in the *Phaeophleospora/Colletogloeopsis* clade (data not shown, TreeBASE SN3058), which is the focus of this study, and thus data were combined as suggested previously (Hognabba & Wedin 2003). The combined data set contained no significant (P<0.01 gl= -090) phylogenetic signal compared to 1000 random trees. Initial heuristic searches of unweighted characters in PAUP resulted in a single most parsimonious trees of 898 steps (CI=0.63, RI=0.81).

Phylogeny generated from the combined ITS and EF-1α data (Figure 1, TreeBASE, SN3058) indicates that *Phaeophleospora* and *Colletogloeopsis* species from eucalypts, including isolates of two unknown taxa, resided in a strongly supported clade, clearly separate from *P. eugeniae*. Furthermore,

species of *Phaeophleospora* (long 0-multiseptate conidia) and *Colletogloeopsis* (short 0-1 septate conidia) were intermixed within the clade with highly supported bootstrap values. Both Bayesian analysis and parsimony analysis place all *Phaeophleospora* spp. and *Colletogloeopsis* spp. together in a strongly supported clade.

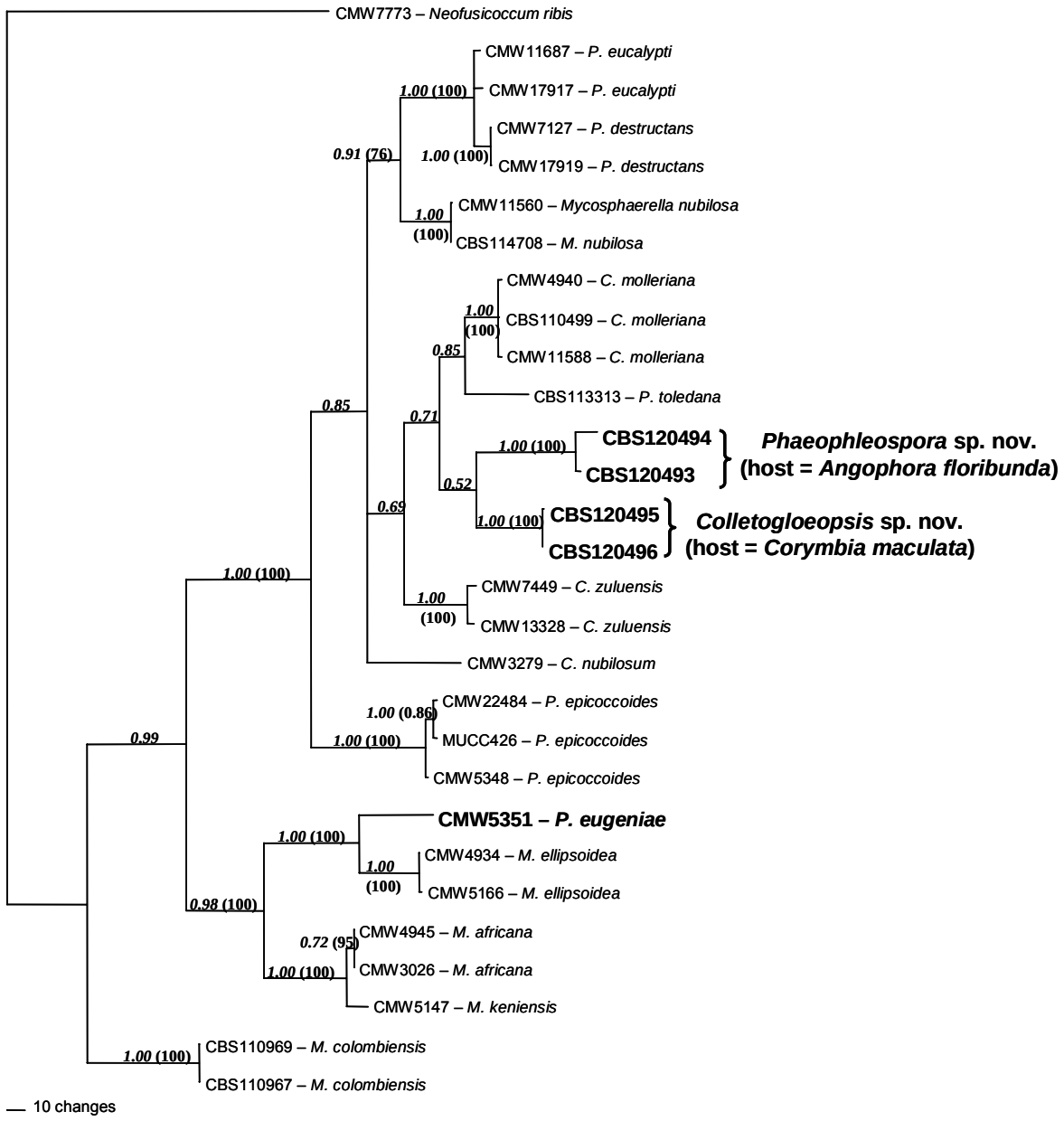


Figure 1. Consensus phylogram of 9500 trees resulting from Bayesian analysis of the combined ITS and EF-1a sequence data for isolates of *Phaeophleospora* and *Colletogloeopsis*. Posterior probabilities of the branch nodes are indicated in italics and bootstrap values resulting from parsimony analysis are indicated in brackets. Two new *Kirramyces* species and the type specimen for genus *Phaeophleospora* are in bold. The tree is rooted to *Neofusicoccum ribis.*

Morphological comparisons

Re-examination of the type specimen of *P. eugeniae* in this study has shown conidia of *P. eugeniae* to differ in conidial pigmentation, length, width and septa number from other *Phaeophleospora* species. While conidia of *P. eugeniae* show variation in pigmentation along the conidial length, ranging from light-brown cells near the base to sub-hyaline cells at the apex, the conidia of other *Phaeophleospora* species are uniformly pigmented. Also, conidia of *P. eugeniae* are much longer, wider and have a greater number of septa when compared to conidia of other *Phaeophleospora* spp. (Table 2).

Morphological observations from herbarium specimens agreed well with the published descriptions although there were minor exceptions (Table 2). Observation of the type specimen of *P. eugeniae* in the present study showed conidia were slightly shorter (100-115 μm) and had less septa (18-20) than those described previously in the literature (110-120 μm, 16-30 septa) (Crous *et al* 1997a). Specimens of *P. destructans* and *P. eucalypti* showed high levels of variability in conidial length, depending on the origin of the specimen. For example, specimens of *P. destructans* from China had shorter conidia (38-47 μm) than those from Indonesia (49-55 μm). The specimen of *P. eucalypti* from Queensland (Australia) had slightly longer conidia (42-47 μm) than *P. eucalypti* from New South Wales (Australia) (38-46 μm). Specimens of *P. destructans* had shorter conidia (38-47 μm) than previously recorded (50-65 μm) by Wingfield *et al.* (1996). *Phaeophleospora epicoccoides*, *P. eucalypti* and *P. destructans* produced shorter conidia *in vitro* than *in vivo*.

Taxonomy

Based on phylogenetic analyses and morphological observations it is clear that *P. eugeniae* is not related to other species of *Phaeophleospora* that are found on eucalypts. Although it has conidia that are peripherally similar to other species of *Phaeophleospora,* they are much longer, broader and more abundantly septate. Re-examination of type specimens in this study has shown that pigmentation is uniform along the length of conidia in all species of *Phaeophleospora* occurring on eucalypts. This is different to *P. eugeniae,* where there is distinct gradation in conidial pigmentation from light brown basal cells to sub-hyaline apical cells. Moreover, phylogenetic analysis has shown that taxa of

Colletogloeopsis from eucalypts are congeneric with taxa of *Phaeophleospora* from eucalypts. A genus is thus needed to accommodate species of *Phaeophleospora* other than *P. eugeniae*. The most appropriate repository for these species is *Kirramyces,* which we resurrect, with an emended description for species of *Phaeophleospora* occurring on eucalypts.

This study has shown that anamorphs of *Mycosphaerella* from eucalypt leaves and stems, currently residing in *Colletogloeopsis*, occur in a single monophyletic assemblage together with species of *Kirramyces*. However, these fungi all have single-celled conidia that are morphologically very different to the multiseptate conidia of *Kirramyces* spp. On the other hand, one of the unknown species emerging from this study, residing in the same phylogenetic group and for which a name is needed, has either aseptate or up to three septate conidia. This implies that there is an obvious gradation from single-celled to multi-septate conidia in the monophyletic lineage that includes species of *Colletogloeopsis* and those species of *Phaeophleospora* known from eucalypts, now shown to be more appropriately accommodated in *Kirramyces*.

Kirramyces as emended to include *Phaeophleospora* and *Colletogloeopsis* species known from eucalypts, produces fruiting bodies that are pycnidial, acervular or both, conidiogenous cells that proliferate percurrently and/or sympodially, and conidia that are rough-walled, or in the case of *Colletogloeopsis* smooth-walled, pigmented, sub-hyaline to medium brown, with 0 or up to 7 septa (Table 3). Thus, the generic description of *Kirramyces* is emended to accommodate additional species with black, erumpent acervuli, cylindrical to sub-cylindrical, sub-hyaline conidiogenous cells and aseptate, fusoid and ellipsoidal, smooth conidia. *Phaeophleospora* is distinguished from *Kirramyces* by the patterns of pigmentation, length of the conidia and number of septa.

Table 2. Morphological features of conidia of *Phaeophleospora, Colletogloeopsis* and *Kirramyces* species from eucalypts recorded in published literature and in the present study. *In vivo* = herbarium specimens, *in vitro* = isolates from culture, n/a=not applicable (the isolates did not produce conidia in culture or were not available.) Schematic drawings are to scale; relative to *Phaeophleospora eugeniae* with a length of 110 µm.

Fungus	Specimen number	Pigmentation	Conidial length (*in vivo*) µm	Conidial length (*in vitro*) µm	Conidial width (*in vivo*) µm	Conidial width (*in vitro*) µm	Number of septa	Schematic drawings of conidia
P. eugeniae (Crous *et al.* 1997a)	IMI 372655	Sub-hyaline to medium brown	110-120	n/a	7-8	n/a	16-30	
Present study	IMI 372655	Versicoloured	100-115	n/a	4-5	n/a	18-20	
P. epicoccoides								
(Walker *et al.*1992)	K 39488	Medium brown	32-50.5	n/a	5-6	n/a	1-4	
(Crous & Wingfield 1997a)	PREM 54963	Medium brown	45-55	40-55	3.5-4	3.5-5	1-7	
Present study	MURU 422	Medium brown	44-50	n/a	3.5-4	n/a	n/a	
	MURU 423	Medium brown	45-48	n/a	3.5-4	n/a	n/a	
	PREM 59260	Medium brown	45-53	36-45	3-4	3-4.5	3-6	
	PREM 59258	Medium brown	41-49	n/a	3-4.	n/a	n/a	
	DAR 6338	Medium brown	34-48	n/a	3.5-5	n/a	n/a	
P. eucalypti								
(Walker *et al.* 1992)	K(M) 39487	Pale brown	35-50	n/a	3-4	n/a	0-2	
Septoria normae (Heather 1961)	DAR 65742	Hyaline, yellow to light brown	24-57		3-3.5		1-2	
Present study	MURU 425	Pale brown	42-47	25-36	2-3	2-3.5	0-3	
	MURU 424	Sub-hyaline	38-46	22-28	2-3	2-3	0-3	
	DAR 65742	Sub-hyaline	35-46	n/a	2-3	n/a	1-2	
P. destructans								
(Wingfield *at al.* 1996)	PREM54416	Pale brown	50-65	n/a	2.5-3	n/a	1-3	
Present study	PREM 59261	Pale brown	38-47	35-40	2-2.5	2-3	1-3	
	PREM 59259	Pale brown	49-55	33-40	2-2.5	2-2.5	1-3	
K. corymbiae Present study	DAR 77445	Pale brown	17-23	16.5-22	3.5-5	2.5-3.5	0	
K. angophorae Present study	DAR 77452	Sub-hyaline to pale brown	9-15	10.5-22.5	2.5-4	3-4.5	0-3	

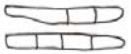
Fungus	Specimen number	Pigmentation	Conidial length (*in vivo*) μm	Conidial length (*in vitro*) μm	Conidial width (*in vivo*) μm	Conidial width (*in vitro*) μm	Number of septa	Schematic drawings of conidia
P. lilianiae								
(Walker *et al.* 1992)	DAR 3833	Medium brown	35-50	n/a	4-6	n/a	1-3	
Present study	DAR 3832	Medium brown	40-48	n/a	5-6	n/a	1-3	
	DAR 3833	Medium brown	35-43	n/a	5-7	n/a	1-3	
P. delegatensis (Park & Keane 1984)	DAR 45718b	Hyaline	21-51	n/a	3-5	n/a	1	
P. toledana (Crous *et al.* 2004)	CBS 59896	Medium brown	10-12	n/a	3-3.5	n/a	0	
C. nubilosum (Crous & Wingfield 1997b)	PDD 37677	Medium brown	10-15	n/a	4-5	n/a	0	
C. molleriana (Crous & Wingfield 1997b)	PREM 54395	Medium brown	9-12	n/a	3-3.5	n/a	0	
C. zuluensis (Cortinas *et al*. 2006b)	IMI 370886	Sub-hyaline to pigmented	4.5-5	n/a	2-2.5	n/a	0-1	
C. gauchensis (Cortinas *et al*. 2006c)	CBS 19722	Medium brown	5-6	n/a	2.5	n/a	0	
C. stellenboschiana (Crous *et al.* 2006)	CBS H-19688	Medium brown	7-9	n/a	3.5	n/a	0	

Table 3. Comparison of morphological characters defining *Phaeophleospora*, *Kirramyces* and *Colletogloeopsis*.

Morphological Characters	***Phaeophleospora*** (Crous *at al.* 1997a)	***Colletogloeopsis*** (Crous & Wingfield 1997b) (Cortinas *et al*. 2006b)	***Kirramyces* (present study)**
Conidiomata	Pycnidial	Pycnidial, acervular	Pycnidial, acervular
Conidiogenous cell	Pigmented, cylindrical to ampulliform, proliferation percurrent	Sub-hyaline to pigmented, doliiform to sub-cylindrical or somewhat irregular, proliferation percurrent and sympodial	Sub-hyaline to pigmented, ampulliform, doliiform to lageniform, or short cylindrical to sub-cylindrical, somewhat irregular proliferation percurrent and sympodial
Conidia	Pigmented, basal cell light-brown, apical cell pale brown, euseptate, vermiform, long, sub-cylindrical to obclavate, smooth to rough walled	Pigmented, aseptate rarely 1-septate, sub-cylindrical, fusoid to ellipsoidal, smooth to verruculose	Pigmented, 0-7, cylindrical-sub-cylindrical, fusoid to ellipsoidal, rough to smooth walled

Emended description for *Kirramyces* is as follows:

Kirramyces J. Walker, B. Sutton & Pascoe, *Mycological Research* **96**:919 (1992).

Mycelium immersed. *Conidiomata* pycnidioid to acervuloid, immersed to erumpent, brown to black, solitary, unilocular; wall 2-5 cells thick, of brown textura angularis or textura epidermoidea; ostiole central, circular, not papillate. *Conidiogenous cells* discrete or produced on superficial hyphae (when cultivated), ampulliform, doliiform to lageniform or short cylindrical to sub-cylindrical, sub-hyaline to brown, verruculose, with 1-several percurrent or sympodial proliferations, formed from the inner cells of the pycnidial wall. *Conidia* holoblastic, pigmented, aseptate or euseptate, fusoid to cylindrical to long obclavate, ellipsoidal tapered to obtuse apices, bases truncate to subtruncate with a marginal frill, smooth to verruculose.

Type species: ***Kirramyces epicoccoides*** (Cooke & Massee) J. Walker, B. Sutton & Pascoe, *Mycological Research* **96**: 919 (1992).

Teleomorph: *Mycosphaerella suttonii* Crous & M.J. Wingf., *Can. J. Bot.* **75**(5): 783 (1997).

Basionym: *Cercospora epicoccoides* Cooke & Massee apud Cooke, *Grevillea* **19**: 91 (1891).

Synonyms: *Hendersonia grandispora* McAlp., *Proc. Linn. Soc. N.S.W.* **28**: 99 (1903).

Phaeoseptoria eucalypti Hansf., *Proc. Linn. Soc. N.S.W.* **82**: 225 (1957).

Phaeoseptoria luzonensis T. Kobayashi, *Trans. Mycol. Soc. of Japan* **19**: 377 (1978).

Phaeophleospora epicoccoides Crous, F.A. Ferreira & B. Sutton, *S. Afr. J. Bot.* **63**(3):113 (1997).

The following species are thus accepted in *Kirramyces*:

Kirramyces delegatensis (R.F. Park & Keane) Andjic **comb. nov.**

Teleomorph: *Mycosphaerella delegatensis* (R.F. Park & Keane) Crous, *Trans. Br. Mycol. Soc.* **83**(1): 95 (1984).

Basionym: *Stagonospora delegatensis* R.F. Park & Keane, *Trans. Br. Mycol. Soc*, **83**(1): 95 (1984).

Phaeophleospora delegatensis (R.F. Park & Keane) Crous, *Mycol. Mem.* **21**: 51 (1998).

Kirramyces destructans M.J. Wingf. & Crous, *South African Journal of Botany* **62**(2): 325 (1996).

Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.
Synonym: *Phaeophleospora destructans* (M.J. Wingf. & Crous) Crous, F.A. Ferreira & B. Sutton, *S. Afr. J. Bot.* **63**(3): 113 (1997).

Kirramyces eucalypti (Cooke & Massee) J. Walker, B. Sutton & Pascoe, *Mycological Research* **96:** 921 (1992).
Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Basionym: *Cercospora eucalypti* Cooke & Massee apud Cooke, *Grevillea* **18**: 7 (1889).

Synonyms: *Septoria pulcherrima* Gadgil & Dick, *N. Z. J. Bot.* **21**: 49 (1983).

Pseudocercospora eucalypti (Cooke & Massee) Guo & Liu, *Mycosystema* **2**: 234 (1989).

Stagonospora pulcherrima (Gadgil & Dick) H.J. Swart, *Trans. Brit. Mycol. Soc.* **90**: 285 (1988).

Phaeophleospora eucalypti (Cooke & Massee) Crous, F.A. Ferreira & B. Sutton, *S. Afr. J. Bot* **63**(3): 113 (1997).

Kirramyces lilianiae J. Walker, B. Sutton & Pascoe. *Mycological Research* **96**: 921 (1992).

Synonym: *Phaeophleospora lilianiae* (J. Walker, B. Sutton & Pascoe) Crous, F.A. Ferreira & B. Sutton, *S. Afr. J. Bot.* **63**(3): 113 (1997).

Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Kirramyces toledana (Crous & G. Bills), Andjic **comb. nov.**

Synonym: *Phaeophleospora toledana* Crous & G. Bills, *Studies in Mycology* **50**: 208 (2004).

Teleomorph: *Mycosphaerella toledana* Crous & G. Bills

Colletogloeopsis spp. from eucalypts are synonymised with *Kirramyces* spp. and new combinations are proposed as follows:

Kirramyces gauchensis (M.N. Cortinas, Crous & M.J. Wingf.), Andjic, M.N. Cortinas & M.J. Wingf. **comb. nov.**

Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Basionym: *Colletogloeopsis gauchensis* M.N. Cortinas, Crous & M.J. Wingf., *Studies in Mycology* **55:** 143 (2006).

Kirramyces molleriana (Crous & M.J. Wingf.), Andjic & M.J. Wingf. **comb. nov.**

Teleomorph: *Mycosphaerella molleriana* (Thum.) Lindau

Basionym:*Colletogloeopsis molleriana* (Crous & M.J. Wingf.), *Canadian Journal of Botany* **75:** 670 (1997).

Kirramyces nubilosum (Ganap. & Corbin), Andjic **comb. nov.**

Teleomorph: *Mycosphaerella cryptica* (Cooke) Hansf., *Proc. Linn. Soc*. N.S.W. **81**: 35 (1956).

Basionym: *Colletogloeum nubilosum* Ganap. & Corbin, *Trans. Brit. Mycol Soc.* **72:** 237 (1979).

Synonym: *Colletogloeopsis nubilosum* (Ganap. & Corbin) Crous & M.J. Wingf., *Can. J. Bot.* **75:** 668 (1997).

Kirramyces stellenbochiana (Crous) Andjic **comb. nov.**

Synonym: *Colletogloeopsis stellenbochiana* Crous, *Stud.Mycol.* **55**:110 (2006).

***Kirramyces* sp.**

Teleomorph: *Mycosphaerella pseudocryptica* Crous, *Stud.Mycol.* **55**:116 (2006), anamorph as *Colletogloeopsis*

Kirramyces zuluensis (M.J. Wingf., Crous & T.A. Cout.), Andjic & M.J. Wingf. **comb. nov.**

Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Basionym: *Coniothyrium zuluense* M.J. Wingf., Crous. & T .A. Cout., *Mycopathologia* **136:** 142 (1997).

Synonym: *Colletogloeopsis zuluensis* (M.J. Wingf., Crous & T.A. Cout), M.N. Cortinas, M.J. Wingf. & Crous, *Mycol Res* **110**: 233 (2006).

Based on phylogenetic analysis and morphological observations it is clear that the new species isolated from *Angophora* and *Corymbia* should reside in the genus *Kirramyces* and they are described below.

Kirramyces corymbiae Carnegie, Andjic & P.A. Barber **sp. nov.** Figures 2A-C, 3.A-C

MycoBank MB510110

Teleomorph: not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Etymology: Named after the host on which this fungus is found.

Conidiomata pycnidialia amphigena, subepidermalia, solitaria ad raro aggregata, atra, globosa, uniloculata, ad 90 µm diam; paries ex 2 vel 3 stratis texturae angularis constans. *Cellulae conidiogenae* discretae, subhyalinae ad pallide brunneae, doliiformes, 6–13 µm. *Conidia* holoblastica, fusiformia, recta ad plerumque curvata, raro sigmoidea, apice subobtuso, basi truncata, non prominente guttulata, pallide brunneae, aseptata, (14–)17–23(–24) × 3.5–5 µm.

Leaf spots amphigenous, sub-circular to irregular, single to confluent, 1–10 mm diam., yellow-brown with thin green-brown to red-purple margin. *Conidiomata* pycnidial, amphigenous, sub-epidermal, single to occasionally aggregated, black, globose, unilocular, to 90 µm diam; wall of 2–3 layers of *textura angularis. Conidiogenous cells* discrete, sub-hyaline to light brown,

doliiform, 6–13 µm. *Conidia* holoblastic, fusiform, straight to mostly curved, occasionally sigmoidal, apex sub-obtuse, base truncate, not prominently guttulate, pale brown, aseptate, (14–)17–23(–24) × 3.5–5 µm.

Conidial germination on MEA after 24 h: Conidia becoming 1–2-septate, germ tubes growing at an acute angle from both ends of the conidia, each germ tube less than 10 µm long at 24 h.

Typus: Holotype: Australia, New South Wales, Mandalong (native forest), on leaves of *C. maculata,* A.J. Carnegie, 15 Jan. 2003 (DAR 77445; culture ex-type DAR 77445).

Cultures: Colonies slow-growing, 9-14 mm diameter on MEA after 1 month at 25°C in the dark, margin white 5Y 8/1, top dark grey 5Y 3/1, bottom light pink 5YR 8/3, colony sectored. *Conidia*: fusiform, pale brown, straight to mostly curved, aseptate (8.5–)10.5–22.5(–25) x (1.8–)3–4.5(–5)(mean=16.5 x 3.5 µm).

Hosts: C. variegata, *C. maculata*, *C. henryi*.

Geographic distribution: Native forests and plantations in NSW Australia, very common and occasionally damaging.

Additional specimens examined: Kirramyces corymbiae on *C. variegata*, Richmond Range State Forest, Mallanganee, NSW, A.J. Carnegie, 22 June 2002 (DAR 77447); on *C. maculata*, adj. Bains Dairy Plantation, Kempsey, A. J. Carnegie, 29 May 2003 (DAR 77448); on *C. maculata* Kiwarak State Forest, A.J. Carnegie, 31 May 2003 (DAR 77449); on *C. variegata*, Zuill Plantation, Dilkoon, NSW, A.J. Carnegie, 24 Aug. 2003 (DAR 77450); on *C. maculata,* Ibbot Plantation, Baryugil, NSW, A.J. Carnegie, (DAR 77446).

Kirramyces angophorae Andjic, Carnegie & P.A. Barber **sp. nov.** Figures 2D-F, 4.A-E

MycoBank 510110

Teleomorph not seen but presumed to be a *Mycosphaerella* sp. based on phylogenetic analysis.

Etymology: Named after the host on which this species was found.

Conidiomata pycnidialia amphigena, plerumque, hypophyllosa, solitaria, atrobrunnea ad atra, uniloculata, ad 92 µm diam. parietibus 3 – stratis texturae angularis. *Cellulae conidiogenae* cellulis superis stromatum orientes, doliiformes ad subcylindraceae vel ampuliformes, aseptatae ad 1-septatae, 6.5–12 x 2.5–4 µm, parietibus crassis, subhyalinae ad pallide brunneae, verruculosae, enteroblasticae prolificantes, 1-3 percurrenter, raro sympodialiter. *Conidia* solitaria, aseptata ad 1-3 septata, subhyalina et pallide brunneae, verrucolosa, fusiformia, subcylindraceae ad ellipsoidea, recta ad parvum curvata, basis truncata, fimbriata, margine imbricato, apice subobtuso ad obtuso, (4.5–)9–15(–19) x (1.5– 2.5–4 (–4.5) (mean=12 x 3.5µm).

Leaf spots amphigenous, circular to irregular, 2-8 mm diam., single to confluent, red-brown with prominent purple border. *Conidiomata* pycnidial, amphigenous, predominantly hypophyllous, solitary, dark brown to black, unilocular, up to 92 µm diam; wall of 3 layers of *textura angularis*. *Conidiogenous cells* arising from upper cells of the stroma, doliiform to sub-cylindrical or ampulliform, aseptate to 1- septate, 6.5–12 x 2.5–4 µm, thick-walled, sub-hyaline to pale brown, verruculose,

proliferating enteroblastically, 1-3 times percurrently, occasionally sympodially. *Conidia* single, aseptate to 1-3 euseptate, subhyaline to pale brown, verruculose, fusoid, sub-cylindrical to ellipsoidal, straight to slightly curved; base truncate with a marginal frill, apex sub-obtuse to obtuse, (4.5–)9–15(–19) x (1.5–)2.5–4(–4.5) (mean=12 x 3.5μm).

Cultures: Colonies 28 x 22 mm after 1 month at 25°C in the dark, produce red pigmentation (10R 5/6) in agar, upper surface of culture olive 5Y 5/3, reverse dark olive 5Y 3/2. *Conidiogenous cells* 5.5–1.6 x 2.5–6 μm. *Conidia* (8.5–)10.5–22.5(–25.5) (mean=16.5 μm) x (1.78–)3–4.5(5–) (mean=3.5 μm) 0-3 euseptate, lateral branches present as secondary conidia, mycelium in culture producing a synanamorph resembling chlamydospores. *Chlamydospores* (9.5–)10–13(–13.5) x (7.5–)9–11.5(–14) (mean=11.5 x 10.5 μm), dark brown, rounded, thick-walled.

Typus: Holotype: on leaves of *Angophora floribunda,* Lane Cove Bushland, Greenwich NSW, Australia, A.J. Carnegie, 27 Feb. 2005 (MURU 426, DAR 77452, culture ex-type DAR 77452).

Hosts: Angophora floribunda.

Geographic Distribution: Native forests in NSW Australia.

Notes: Kirramyces angophorae can be distinguished from other *Kirramyces* spp. by producing a synanamorph with chlamydospore-like structures and a red pigment in culture. Unlike other *Kirramyces* spp., *K. angophorae* produces longer conidia in culture than on the host.

Additional specimens examined: Kirramyces angophorae on *Angophora floribunda* Raymond Terrace, NSW, Australia, A.J. Carnegie, 29 Nov. 2005 (DAR 77451).

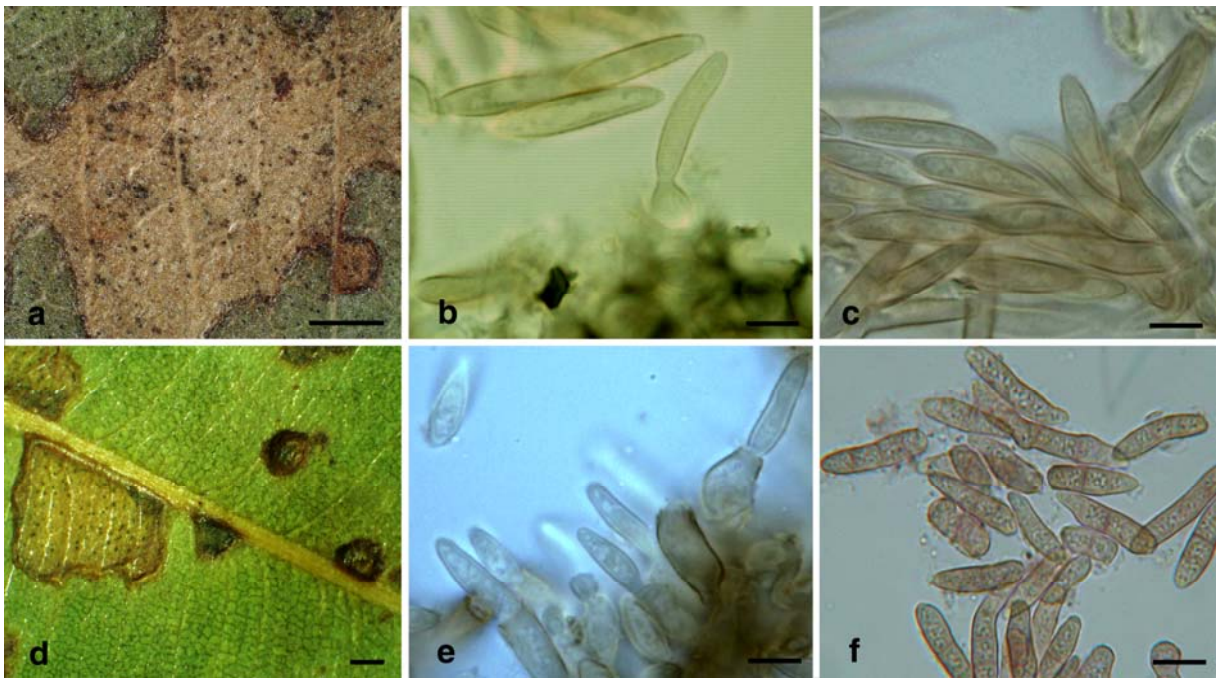


Figure 2. *Kirramyces corymbiae* (a). Leaf lesion on *Corymbia maculata* showing small black pycnidia (b). Conidium attached to a conidiogenous cell, (c). Aseptate conidia; *Kirramyces angophorae* (d). Leaf lesion on *Angophora floribunda* showing small black pycnidia (e). Conidia attached to conidiogenous cells, (f). Aseptate to multi-septate conidia. Scale bar a, d = 10 mm, Scale bar b, c, e, f =10 μm. Squash mounts prepared from *in vivo* material.

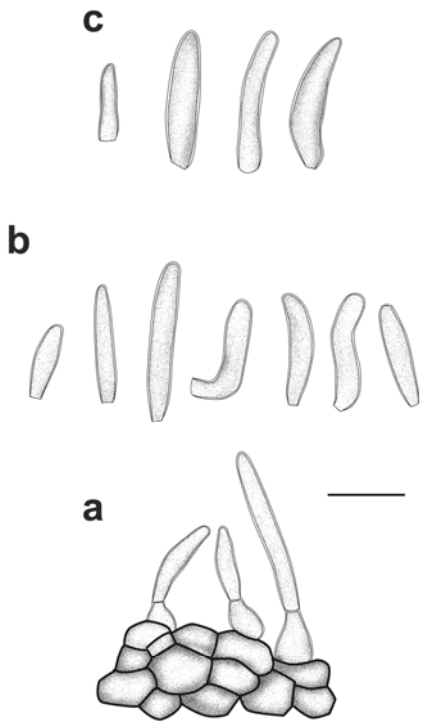


Figure 3. *Kirramyces corymbiae* (a-b) conidiogenous cells and conidia produced *in vivo*. (c) conidia produced on MEA. Scale bar = 10 μm.

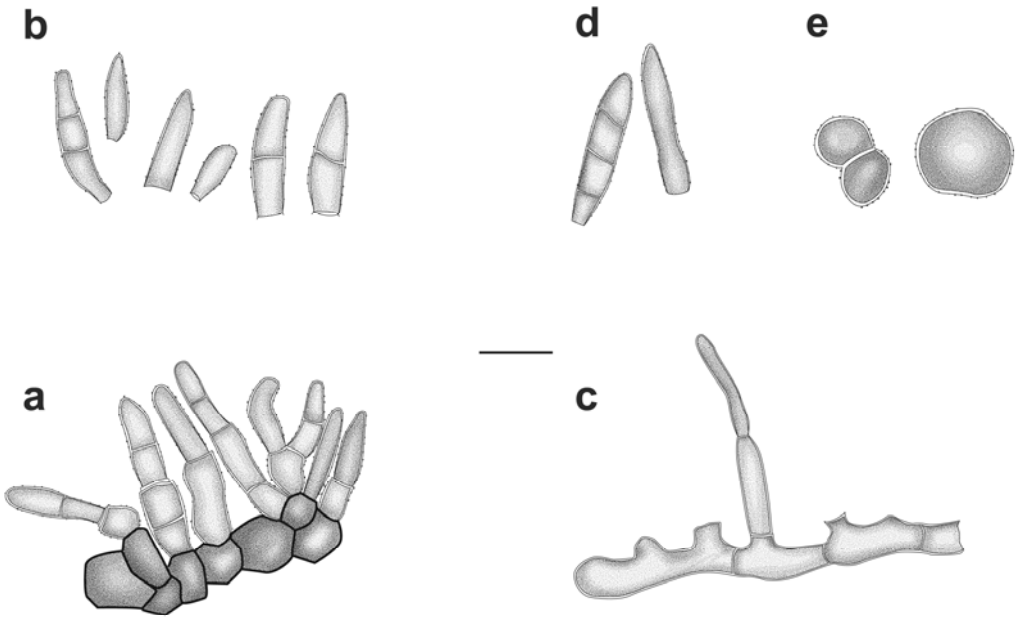


Figure 4. *Kirramyces angophorae.* (a-b), conidiogenous cells and conidia produced *in vivo*, (c-d), conidiogenous cells and conidia produced on MEA, (e) mycelium in culture producing chlamydospore- like synanamorph. Scale bar = 10 μm

Key to Kirramyces *species occurring* on eucalypts

1. Conidia versicoloured, apex and basal cells lighter than the rest of conidial body, on average >100 µm long…...**Phaeophleospora**
   Conidia uniformly pigmented, on average <70 µm long……..………………………………………………...…….**Kirramyces**

2. Conidia aseptate to rarely 1-septate...…………………….……….……………………………………..……………….……….3
   Conidia 1 to multi-septate, rarely aseptate…………..………………………………………………………..…………….………10

3(2) Conidia aseptate, fusiform, straight to mostly curved, occasionally sigmoidal, apex sub-obtuse, base truncate,
Conidia on average >17µm in length…………...………………………………………………………………...…….**corymbiae**
Conidia on average < 15µm in length……………..………………...…………………...………………….………..……….4

4(3) Conidia on average < 6 µm in length…………..…………..…………..…………………………..………………………...………..5
Conidia on average > 6 µm in length……………....…..…………….…………………....……………………….……..……...6

5(4) Conidia broadly ellipsoidal, finely verruculose, apex obtuse to sub-obtuse, base sub-truncate to bluntly rounded,
5-6 x 2.5 µm………………………………………………………...…………..………………….……….…..…**gauchensis**
Conidia, fusoid to subcylindrical to ellipsoidal, smooth to verruculose, apex obtuse, base, subtruncate,
4.5-5x 2-2.5 µm……………….……………………………………….……...……………………….………...…..**zuluensis**

6(4) Conidia on average <10 µm in length………………………..…….………………………………..……….........**stellenboschiana**
Conidia on average > 10 µm in length………………………..…………………..…..…...…………………..…….……...…...7

7(6) Conidia fusoid………………………………..……………………..……….……….…………….……..…………….……..8
Conidia subcylindrical to ellipsoidal……………………………..………..………...9

8(7) Conidia, finely verruculose, 12-14 x 4 µm; teleomorph*Mycospharella pseudocryptica*......................................**Kirramyces sp.**
Conidia, verruculose, apex acutely rounded, base truncate with a minute marginal frill, 10-12 x 3-3.5 µm; teleomorph *Mycosphaerella toledana*……………………………………………………………………………………….………**toledana**

9(7) Conidia, aseptate rarely becoming 1-septate in culture, 9-12 x 3-3.5 µm; teleomorph *Mycosphaerella molleriana*..**molleriana**
Conidia, aseptate, 10-15 x 4-5 µm; teleomorph *Mycospharella cryptica* ………………………………..………….**nubilosum**

10(2) Conidia on average <30 µm in length,…………………………………………………………………………….…..**angophorae**
Conidia on average > 30 µm in length……………………………………………...11

11(10) Conidia medium brown, typically 3-5 septate……………………………….……………………………………….…….12
Conidia hyaline to sub-hyaline to pale brown, typically 1-3 septate ……………….……..……..13

12(11) Conidia typically 3-5-septate, occasionally with up to 7 septa,subcylindrical to narrowly obclavate, apex sub-obtuse 45-55x 3.5-4µm; teleomorph *Mycosphaerella suttonii* ……………………………………………………………………….**epicoccoides**
Conidia typically 3-septate, cylindrical, apex obtuse, 40-48 x 5-6 µm, no known teleomorph ……………………..…..…**lilianiae**

13(11) Conidia 1-septate, hyaline, cylindrical, straight or curved, smooth, thin walled, apex obtuse, base truncate 21-51 x 3-5 µm; teleomorph *Mycosphaerella delegatensis*……..……………………………………………………………….…...**delegatensis**
Conidia subhyaline to pale brown……………….……………………………………………...…………………………......14

14(13) Conidia typically 1-2-septate, less typically 0-3-septate, subcylindrical to narrowly obclavate, thick walled, finely verruculose, apex sub-obtuse, inconspicuous marginal frill present 35-50 x 3-4 µm, no known teleomorph………....**eucalypti**
Conidia 1-3-septate, cylindrical, verruculose, apex obtuse, marginal frill mostly absent 50-65 x 2.5 µm, no known teleomorph.……………………………………..**destructans**

DISCUSSION

Mycosphaerella spp. and their anamorphs include some of the most important pathogens of eucalypts. Many of them have also been moved around the world through the establishment of plantations of these trees. In recent years, numerous new species of these fungi have been described (Crous *et al*. 2004, 2006) and based on the large number of eucalypt species, it seems likely that many more will be discovered in the future. Many of these fungi are morphologically similar or difficult to distinguish based on morphology and their contemporary taxonomy relies heavily on DNA sequence comparisons. Results of this study, using phylogenetic inference and morphological characteristics, have led to the discovery of two new species of these fungi. In order to accommodate these species, the need to modify the boundaries of the genera to which they belong became evident.

The present phylogenetic and morphological study has shown the type specimen of the genus *Phaeophleospora*, *P. eugeniae* is well separated from *Phaeophleospora* spp. occurring on eucalypts. This phylogenetic separation logically led to the resurrection of the previous generic name, *Kirramyces*, for these species. Furthermore, phylogenetic analysis combined with the overlapping morphological characters of *Kirramyces* spp. and *Colletogloeopsis* spp. occurring on eucalypts, supporting the synonymy of these genera. Thus, anamorphs of *Mycosphaerella* residing in *Phaeophleospora* occurring on eucalypts, as well as species of *Colletogloeopsis*, have been transferred to the newly resurrected genus *Kirramyces*. Re-examination in the present study of the type specimens of both *Phaeophleospora* and *Kirramyces* has shown that variation in pigmentation of conidia is a useful morphological feature in distinguishing between these two genera.

The phylogenetic relationship between the genera *Phaeophleospora* (now *Kirramyces*) and *Colletogloeopsis* has been shown in previous studies (Chapter 2; Cortinas *et al.* 2006c; Crous *et al.* 2001, 2006; Hunter *et al.* 2006). The two genera have not previously been combined, mainly because *Phaeophleospora* produced pycnidioid conidiomata and 0-multiseptate conidia, whereas *Colletogloeopsis* produced acervular conidiomata and aseptate or rarely 1-septate conidia. The emendment of the description of *Colletogloeopsis* to accommodate species with pycnidia (Cortinas *et al.* 2006b) resulted in conidial size and septation being the only morphological characters separating

the two genera. However, discovery of new species such as *K. angophorae*, with aseptate as well as multiseptate conidia, precludes retaining *Colletogloeopsis* for aseptate species in this group. Furthermore, these differences are not phylogenetically supported between species within the genus.

In the present study, it was possible to obtain the sequences for the ITS1 region from the type specimens of *K. lilianiae* and *K. delegatensis.* Walker *et al*. (1992) described *K. lilianiae* as morphologically very similar to *K. epicoccoides*. However, due to the lack of a suitable number of collections of *K. lilianiae*, comparison between the two species was not possible. *Stagonospora delegatensis*, first described by Park & Keane (1984) was later considered by Swart (1988) to be congeneric with *Septoria pulcherrima*. Walker *et al*. (1992) noted that *S. delegatensis* was similar to *K. eucalypti* and thus was a possible candidate to transfer to *Kirramyces*, but required further collections. It was later reduced to synonymy with *K. eucalypti* (Sankaran *et al.* 1995). Subsequently, Crous (1998) re-examined the type specimen of *S. delegatensis* and supported the placement of this fungus in the genus *Kirramyces*. However, based on conidial shape, they chose to retain the species separate from *K. eucalypti* and transferred it to *Phaeophloeospora* as *P. delegatensis*. Results of the present study based on ITS1 sequence data have shown that *K. delegatensis* and *K. lilianiae* cluster together with other *Kirramyces* species occurring on eucalypts, therefore confirming its placement in *Kirramyces*.

Kirramyces was originally described for three species: *K. epicoccoides*, *K. eucalypti* and *K. lilianiae.* Based on the results of the present and previous studies, the genus now includes fourteen species. These all reside in a well resolved monophyletic clade based on DNA sequence comparisons. They also have conidia ranging from those that are aseptate to multiseptate.

Mycosphaerella Johanson is a heterogenous genus that is linked closely to a large number of anamorphs that lack known teleomorphs (Crous & Braun 2003). Previous authors have debated whether anamorphic states should be used to separate genera, subgenera or sections within *Mycosphaerella*. Sutton & Hennebert (1994) held the view that different conidiogenous events and conidiomatal types in anamorphs linked to *Mycosphaerella* may prove useful in grouping species at

some sub-generic level. Based on phylogeny, this has not proved to be true, as many anamorphs of *Mycosphaerella* spp. are currently polyphyletic (Crous *et al.* 2006).

Hunter *et al.* (2006) suggested that anamorph relationships based on phylogenetic position within *Mycosphaerella* cannot be predicted. However, results of the present study have shown that all *Kirramyces* spp. from eucalypts (including several as yet undescribed species) reside in the same strongly supported clade. This is also true for species of *Pseudocercospora* (Crous & Braun 2003) and *Readeriella* Syd. & P. Syd. (Crous *et al.* 2004, 2006; Hunter *et al.* 2006). Morphologically, *Readeriella* spp. are somewhat similar to species in the genus *Kirramyces,* but are distinctly different as *Readeriella* spp. have obvious phialidic conidiogenesis. This was a key feature used by Sutton (1980) to differentiate between the genera *Microsphaeropsis* Höhn. and *Coniothyrium* Corda occurring on eucalypts. A number of species collected and identified as *Microsphaeropsis* or *Kirramyces,* based on morphological characters, have subsequently been compared based on DNA sequence data and these have resolved taxonomic conflicts between *Readeriella* and *Kirramyces* (Andjic, unpub. data). In the case of *Readeriella* and *Kirramyces*, the mode of conidiogenesis appears to be phylogenetically significant. We suspect that new collections and subsequent DNA sequence comparisons for previously described *Microsphaeropsis* spp. from eucalypts, including *M. conielloides* B. Sutton, *M. callista* (Syd.) B. Sutton, *M. globulosa* (Sousa da Câmara) B. Sutton and *M. olivaceae* (Bonord.) Höhn. will show that these fungi reside in the genus *Readeriella.*

Data emerging from this study provide clear evidence that at least some groups of anamorphs of *Mycosphaerella* spp. reside in strongly monophyletic lineages. These are generally also consistent with their morphological features. These are interesting and important observations that most likely reflect ecological adaptation and evolutionary events. How these relate to a possible subdivision of *Mycosphaerella* based on phylogenetic inference is difficult to predict. Clearly, many anamorph genera are emerging in discrete clades that are very different to the one that accommodates *M. punctiformis* (Pers.) Starbäck, the type species of the genus. There is good evidence that *Mycosphaerella* is polyphyletic and its subdivision into more natural subdivisions will emerge in time.

The anamorphs of this important genus encompass valuable ecological inference and should not be lost from future phylogenetic treatments of the group.

This study has clarified the generic placement of a large number of *Mycosphaerella* spp. or their anamorphs occurring on leaves, shoots and stems of eucalypts. These also include some of the most important pathogens of *Eucalyptus* residing in *Mycosphaerella*. For example, the stem pathogens, *K. zuluensis* and *K. gauchensis* cause the disease known as *Coniothyrium* canker, which is one of the most important diseases of *Eucalyptus* spp. grown in plantations (van Zyl 1999; Wingfield *et al.* 1997). Likewise *K. destructans*, *K. eucalypti*, *K epicoccoides* and *K. nubilosum* (anamorph of *M. cryptica*) represent four of the most important leaf pathogens of *Eucalyptus* spp. (Park *et al*. 2000; Barber 2004; Burgess *et al.* 2006a; Carnegie 2007b). Of these, *K. destructans* is particularly damaging because it infects both leaves and shoots of trees and it has caused substantial damage to plantations in south-east Asia. The majority of the most important *Mycosphaerella* spp. that infect eucalypts reside in the phylogenetic clade accommodating species of *Kirramyces*. The evolutionary significance of this relationship deserves further study.

CHAPTER 4

Anthropogenic movement of the serious *Eucalyptus* leaf-blight pathogen *Kirramyces destructans* throughout South-East Asia

ABSTRACT

Kirramyces destructans is a serious pathogen that causes leaf, bud and shoot blight diseases of *Eucalyptus* spp. in plantations in the sub-tropics and tropics of South East Asia. This pathogen was first discovered in Indonesia in 1995 and has subsequently spread to Thailand, China, Vietnam and East Timor. Very little is known regarding the biology, ecology and genetics of this important pathogen. The objective of this study was, thus, to determine the genetic diversity and movement of *K. destructans* throughout south-east Asia using multi gene phylogenies and microsatellite markers. While two microsatellite markers detected a very low nucleotide polymorphism between isolates, eight other gene regions, including four microsatellite regions, reflected genetic uniformity. This low level of genetic diversity suggests that *K. destructans* was introduced into Indonesia as a founder population from which it has subsequently spread throughout Asia via human-mediated movement of germplasm.

INTRODUCTION

Kirramyces destructans is an aggressive and often devastating pathogen that causes blight of young leaves, buds and shoots of some *Eucalyptus* spp. This pathogen was first discovered in Northern Sumatra, Indonesia where it caused serious damage to young leaves of *E. grandis* (Wingfield *et al.* 1996). Since then, *K. destructans* has appeared in Thailand, China and Vietnam and East Timor. Disease occurs on *E. camaldulensis*, *E. urophylla*, *E. grandis* and various hybrids between these three species (Burgess *et al.* 2006a; Old *et al.* 2003a, 2003b). *Eucalyptus* spp. are non-native in most of these countries where they have been used to establish plantations for fibre production. The exception is East Timor, where *K. destructans* was found on native *E. urophylla* (Old *et al*. 2003b).

Whilst *K. destructans* and the disease it causes was first discovered in Northern Sumatra, the origin of the pathogen is unknown. During surveys of eucalypt diseases in Mareeba (northern Queensland), Tiwi Island (Northern Territory) and the Kimberly (Western Australia), various undescribed *Kirramyces* spp. have been discovered (Appendix III). These include *K. destructans* (Burgess *et al*. 2007b) and the recently described pathogen *Kirramyces viscidus* (Chapter 6), which is closely related to *K. destructans*. In addition, earlier collections from East Timor have been confirmed to be *K. destructans*, after the extraction DNA from the herbarium material (Morag Glen, pers. comm.). Timor is only 500 km north of Australia, in addition to historical trade between indigenous peoples (Clarke 2003); strong winds during monsoonal periods (Vallgren 2006) could potentially blow fungal spores in either direction. It is likely that *K. destructans* is native to both northern Australia and Timor or has been established in both regions for some time and was accidentally introduced to Indonesia, perhaps on infected germplasm or seed during the many seed collection expeditions that have been made to Timor to collect seed of *E. urophylla*.

Regardless of the origin of the pathogen, it has been moved rapidly throughout the region. Thus, the aim of this study was to use microsatellite markers to follow the pathway of its movement throughout Asia of *K. destructans* throughout South-East Asia and China. In addition, five gene regions were sequenced and analysed.

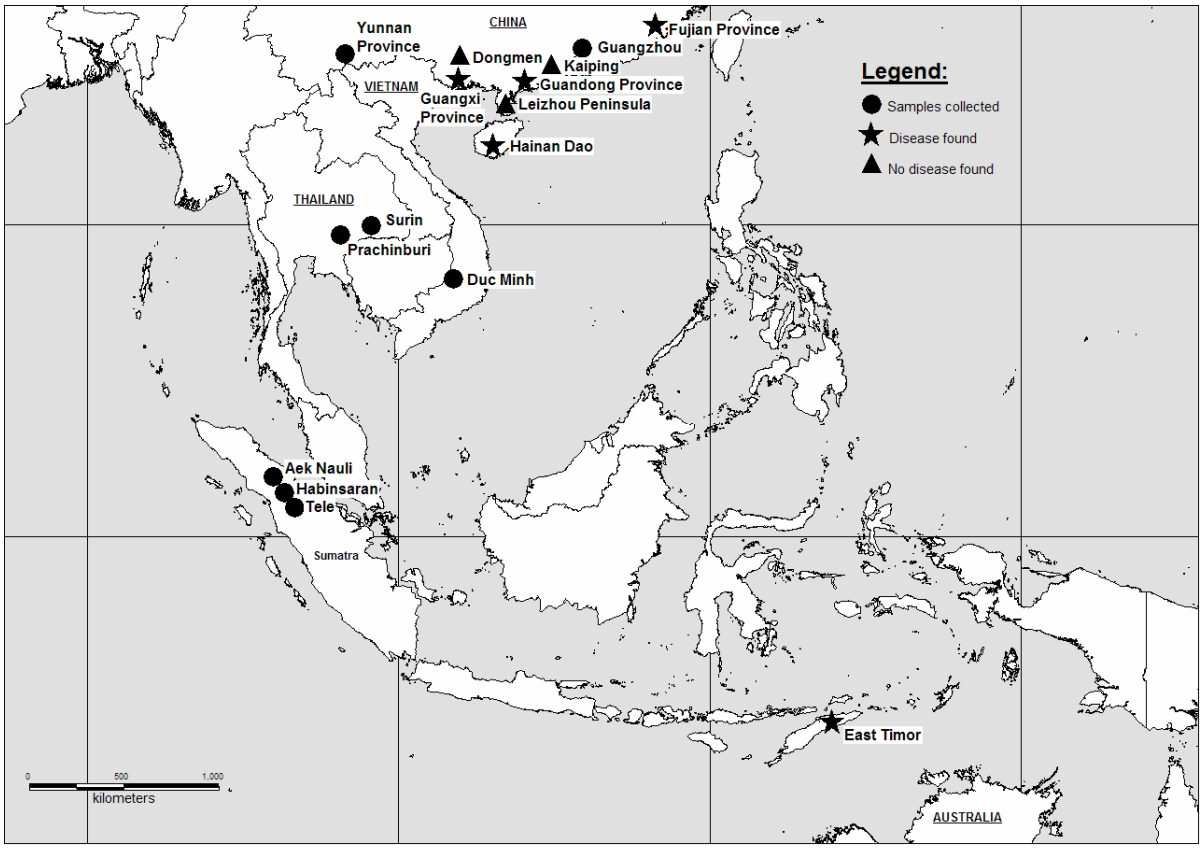


Figure 2. Map showing the distribution of localities where leaf material infected with *K. destructans* has been observed and collected.

MATERIALS AND METHODS

Fungal isolates and DNA extraction

Eucalyptus leaves infected with *K. destructans* (Figure 1) were collected from South East Asian countries including Indonesia, China, Vietnam and Thailand on separate visits between 2003-2005 (Figure 2). *Kirramyces destructans* isolates were isolated and cultures maintained as described in Chapter 2. After 4 weeks, the mycelium was harvested and placed in 1.5 ml sterile Eppendorf® tubes. Harvested mycelium was frozen in liquid nitrogen, ground to a fine powder and genomic DNA extracted as described previously (Chapter 2). All isolates are maintained in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Forty-three representative isolates, out of a collection of 300 isolates from a range of geographical locations and hosts were selected for study. These included 12 from Thailand, three from Vietnam, nine from China and 19 from Indonesia (Table 1).

Figure 1. Leaf symptoms infected with *Kirramyces destructans*; a) A young *Eucalyptus grandis* x *E. urophylla* seedling from Indonesia with severe infection, b) spore mass on the abaxial leaf surface.

Table 1. *Kirramyces destructans* isolates considered in this study.

Culture no.[1]	Host	Location	Year	Collector
CMW 19855	*E. grandis* x *E. urophylla*	Tele, Indonesia	2003	PA Barber
CMW 19892	*E. grandis*	Aek Nauli, Indonesia	2004	P Dolok Saribu
CMW 19854	*E. grandis* x *E. urophylla*	Tele, Indonesia	2003	PA Barber
CMW 19864	*E. grandis* x *E. urophylla*	Aek Nauli, Indonesia	2004	P Dolok Saribu
CMW 19886	*E. grandis*	Aek Nauli, Indonesia	2004	P Dolok Saribu
CMW 19887	*E. grandis*	Aek Nauli, Indonesia	2004	P Dolok Saribu
CMW 19860	*E. grandis* x *E. urophylla*	Habinsaran, Indonesia	2003	PA Barber
CMW 19851	*E. grandis* x *E. urophylla*	Habinsaran, Indonesia	2003	PA Barber
CMW 19850	*E. grandis* x *E. urophylla*	Habinsaran, Indonesia	2003	PA Barber
CMW 19835	*E. grandis* x *E. urophylla*	Tele, Indonesia	2003	PA Barber
CMW 19852	*E. grandis*	Aek Nauli, Indonesia	2003	PA Barber
CMW 19837	*E. grandis* x *E. urophylla*	Tele, Indonesia	2003	PA Barber
CMW 19853	*E. grandis*	Aek Nauli, Indonesia	2003	PA Barber
CMW 19842	*E. grandis* x *E. urophylla*	Tele, Indonesia	2003	PA Barber
CMW 19845	*E. grandis* x *E. urophylla*	Tele, Indonesia	2003	PA Barber
CMW 19891	*E. grandis*	Aek Nauli, Indonesia	2004	P Dolok Saribu
CMW 19832	*E. grandis* x *E. urophylla*	Tele, Indonesia	2003	PA Barber
CMW 19831	*E. grandis* x *E. urophylla*	Tele, Indonesia	2003	PA Barber
CMW 19834	*E. grandis* x *E. urophylla*	Tele, Indonesia	2003	PA Barber
CMW 13705	*E. camaldulensis*	Tatoom, Thailand	2003	MJ Wingfield
CMW 13337	*E. camaldulensis*	Tatoom, Thailand	2003	MJ Wingfield
CMW 13709	*E. camaldulensis*	Tatoom, Thailand	2003	MJ Wingfield
CMW 13330	*E. camaldulensis*	Tatoom, Thailand	2003	MJ Wingfield
CMW 13710	*E. camaldulensis*	Tatoom, Thailand	2003	MJ Wingfield
CMW 16136	*Eucalyptus* sp.	Prachinburi, Thailand	2003	MJ Wingfield
CMW 16124	*Eucalyptus* sp.	Prachinburi, Thailand	2003	MJ Wingfield
CMW 16123	*E. camaldulensis*	Prachinburi, Thailand	2003	MJ Wingfield
CMW 16120	*Eucalyptus* sp.	Prachinburi, Thailand	2003	MJ Wingfield
CMW 16138	*Eucalyptus* sp.	Prachinburi, Thailand	2003	MJ Wingfield
CMW 16137	*Eucalyptus* sp.	Prachinburi, Thailand	2003	MJ Wingfield
CMW 16126	*Eucalyptus* sp.	Prachinburi, Thailand	2003	MJ Wingfield
CMW 15089	*E. camaldulensis*	MinhDuc, S-E Vietnam	2005	TI Burgess
CMW 15090	*E. camaldulensis*	MinhDuc, S-E Vietnam	2005	TI Burgess
CMW 15092	*E. camaldulensis*	MinhDuc, S-E Vietnam	2005	TI Burgess
CMW 19934	*E. urophylla*	Guangdong, China	2005	TI Burgess
CMW 19914	*E. urophylla*	Guangdong, China	2005	TI Burgess
CMW 19911	*E. urophylla*	Guangdong, China	2005	TI Burgess
CMW 19922	*E. urophylla*	Guangdong, China	2005	TI Burgess
CMW 19933	*E. urophylla*	Guangdong, China	2005	TI Burgess
CMW 19921	*E. urophylla*	Guangdong, China	2005	TI Burgess
CMW 19909	*E. urophylla*	Yunnan, China,	2004	B Dell
CMW 19910	*E. urophylla*	Yunnan, China	2004	B Dell
CMW 19908	*E. urophylla*	Yunnan, China	2004	B Dell

[1] Designation of isolates and culture collections: CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

Development of microsatellite markers

To screen for microsatellites, the fast isolation by amplified fragment length polymorphism (AFLP) of sequences containing repeats (FIASCO) technique (Zane *et al*. 2002) was applied using the procedures described by Cortinas *et al*. (2006a). DNA from three isolates (CMW 19866 and CMW 19832 from Indonesia and CMW 19908 from China) was pooled. One μg of genomic DNA was digested with *Mse*I (Biolabs, New England) and ligated to the adaptor using highly concentrated T4 DNA ligase 2 000 000 U/ml (Biolabs, New England). The digestion-ligation mixture was incubated overnight at 37 C° and the reaction inactivated at 65 C° for 20 min. Five μl of the mixture was used for PCR following the method of Cortinas *et al*. (2006a). After amplification, PCR products were hybridised to $(GACA)_5$, $(TCC)_7$, $(CAT)_5$, $(TGC)_4$, $(CA)_{10}$ and $(CT)_{10}$ biotinylated probes. Hybridized DNA complexes were captured on streptavidin magnetic beads (Dynal beads Streptavidin, DYNAL, Biocompare, Inc. San Francisco, CA, USA) and DNA separated by washing and denaturation steps. Recovered DNA was precipitated and amplified using the *Mse*I-N primer.

Amplified DNA was cloned into pGEM®-T Eazy Vector Systems (Promega, Madison, USA) following the manufacturer's instructions. Bacterial clones were selected, amplified, purified and sequenced as described by Cortinas *et al*. (2006a). Primers were developed with the aid of the primer design software PRIMER 3 (Rozen & Skaletsky 2000) available on the Internet at http://frodo.wi.mit.edu. To test for polymorphisms, ten isolates representing a range of geographical origins were chosen (CMW 19855, CMW 19892, CMW 19864, CMW 19837, CMW 19845, CMW 19831 from Indonesia, CMW 19908, CMW19933 from China, CMW 15090 from Vietnam and CMW 16123 from Thailand.

PCR amplification and sequencing

This study included amplification of the complete internal transcribed spacer region 2 (ITS2), part of the β-tubulin gene region (βT), part of the elongation factor 1α gene (EF-1α), part of the chitin synthase 1 gene (CHS), part of the ATPase gene (ATP-6) and the microsatellite markers VA-1, VA-2, VA-6, VA-13, VA-15 and VA-18. Primers used to amplify these regions are listed in Table 2. The PCR reaction mixture (25 μl), PCR conditions and visualisation of products were as described in

Cortinas *et al*. (2006) except for the ATP-6 region, which was amplified using the following conditions: initial denaturation of 7 min at 94 °C, followed by 40 cycles of 45 s at 94 °C, 45 s at 45 °C, 2 min at 65 °C and a final elongation step of 10 min at 68 °C. Where amplifications failed, the magnesium concentration was increased to 4 mM, and the primer concentration were increased to 0.9 *p*mol and the following PCR conditions were used: 7 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 45 °C, 2 min at 72 °C and a final elongation step of 10 min at 72 °C. The PCR products were purified with the Ultrabind®DNA purification kit (MO BIO Laboratories, Solana Beach, CA) following the manufacturer's instructions. Amplicons were sequenced as described previously (Burgess *et al*. 2005). All sequences obtained in this study were deposited in GenBank and accession numbers are given in Table 3.

Table 2. Primer sets and annealing temperature used to amplify *Kirramyces destructans.*

Region	Oligos	Oligo Sequence (5'-3')	Amplicon size (bp)	AT (ºC)	Reference
ITS	ITS-3 ITS-4	GTATCGATGAAGAACGCAGC TCCTCCGCTTATTGATATGC	250	55	(White *et al.*, 1990)
β-tubulin	Bt2a Bt2b	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	680	45 55	(Glass, Donaldson, 1995)
EF-1α	EF1-728F EF1-986R	CATCGAGAAGTTCGAGAAGG TACTTGAAGGAACCCTTACC	350	45-55	(Carbone, Kohn, 1999)
CHS	CHS-79F CHS-354R	TGTGGGCAAGGATGCTTGGAAGAAG TGGAAGAACCATCTGTGAGAGTTG	300	55	(Carbone, Kohn, 1999)
ATP6	ATP6-1 ATP6-2	ATTAATTSWCCWTTAGAWCAATT TAATTCTANWGCATCTTTAATRTA	600	45	(Kretzer, Bruns, 1999)
VA-1	VA1F VA1R	CAGAGATCGCAGCAGTACAG CAGTTGGAGGCAAGGACAAG	267	55	This study
VA-2	VA2F VA2R	CTGCGATTCTGGAAGCTTCG GGCAATGATCTCAATGCGGTC	296	53	This study
VA-6	VA6F VA6R	CTACTTCCTAAGTACCTAAGCC CTAAGCTCTTAGAAGAGCTCG	281	55	This study
VA-13	VA13F VA13R	GTACAGGAACCAGACTTCCTAC GATGCGCCTCACTTCTATCC	294	53	This study
VA-15	VA15F VA15R	CAGGTGATTCGACACAATGC GATGAGTCCTGAGTAAGTTGTGG	321	45	This study
VA-18	VA18F VA18R	CGATGAAGTTGACGATAGGC CATGCGCCACGCACGACCAGG	383	53	This study

Base codes: R (AG), N (AGCT), S (GC), W (AT)

RESULTS

DNA sequence comparisons

Five gene regions were compared for sequence variation in *K. destructan*s. In Chapters 2 and 3 no sequence variation in ITS and βt gene regions between *K. destructans* isolates from Indonesia and China were observed (Table 3). Thus, only the 12 isolates from Thailand and three isolates from Vietnam were sequenced and compared with those already sequenced from Indonesia and China. Where no additional polymorphisms were observed, further isolates were not sequenced. Direct sequencing of PCR amplicons of ATP6, CHS and EF-1α, gene regions from all 43 isolates showed no sequence variation between *K. destructans* isolates.

Microsatellite markers

Six of the 20 primer pairs amplified a microsatellite-containing region for all tested representative isolates of *K. destructans*. Four of the primer pairs (VA-1, VA-6, VA-13, and VA-15) were monomorphic among the isolates tested. Two of the primer pairs (VA-2 containing a TG repeat and VA-18 containing a CCA repeat) showed a low level of polymorphism and were amplified and sequenced for the 43 selected isolates. In addition VA-13, which appeared to be monomorphic, was also sequenced as it contained a very long CCA repeat.

The loci amplified by VA-13 were monomorphic among all 43 isolates. However, the loci amplified by VA-2 and VA-18 showed a low nucleotide polymorphism (Table 3). For microsatellite marker VA-2, three alleles(A-C) were observed among the 19 isolates from Indonesia. The predominant allele (A) was the only allele found among isolates from China, Thailand and Vietnam (Table 3). Three alleles (A-C) were also observed for VA-18 among the 19 isolates from Indonesia. All isolates from China were of allele B, while isolates from Thailand and Vietnam belonged to allele A (Table 3). In combination there were five haplotypes in the Indonesian population (AB, AC, BA, BB, and CA), one haplotype AB in China and one haplotype AA in Thailand and Vietnam. There was no association of alleles among isolates from Indonesia suggesting that outcrossing is occurring even though a

teleomorph has never been observed for this species. While all alleles were found among the Indonesian isolates, the haplotype AA was not observed there.

Table 3. *Kirramyces destructans* Genbank accession numbers. For VA-2 and VA-18, the haplotype for each isolate is given next to the Genbank accession no.

Isolate no.	Location	Genbank Accession no.										
		ITS	ß-tubulin	EF-1α	CHS	ATP6	VA-1	VA-2	VA-6	VA-13	VA-15	VA-18
CMW 19855	Indonesia	EU019888	EU019880	EF686485	EF686308	EF686254	EU620606	EF686353 (C)	EU620611	EF686398	EU620619	EF686443 (A)
CMW 19892	Indonesia			EF686486	EF686309	EF686255	EU620603	EF686354 (A)	EU620612	EF686399	EU620620	EF686444 (C)
CMW 19854	Indonesia			EF686487	EF686310	EF686256		EF686350 (C)		EF686400		EF686445 (A)
CMW 19864	Indonesia			EF686481	EF686304	EF686250	EU620604	EF686349 (B)	EU620613	EF686394	EU620621	EF686439 (B)
CMW 19886	Indonesia	EU019889	EU019881	EF686490	EF686313	EF686259		EF686358 (A)		EF686403		EF686448 (C)
CMW 19887	Indonesia			EF686491	EF686314	EF686260		EF686359 (B)		EF686404		EF686449 (A)
CMW 19860	Indonesia			EF686488	EF686311	EF686257		EF686356 (A)		EF686401		EF686446 (C)
CMW 19851	Indonesia	EU019890	EU019882	EF686497	EF686320	EF686266		EF686365 (A)		EF686410		EF686455 (B)
CMW 19850	Indonesia	EU019895	EU019887	EF686495	EF686318	EF686264		EF686363 (A)		EF686408		EF686453 (C)
CMW 19835	Indonesia			EF686489	EF686312	EF686258		EF686357 (A)		EF686402		EF686447 (B)
CMW 19852	Indonesia			EF686482	EF686305	EF686251		EF686350 (A)		EF686395		EF686440 (C)
CMW 19837	Indonesia	EU019892	EU019884	EF686483	EF686306	EF686252	EU620601	EF686351 (A)	EU620610	EF686396	EU620618	EF686441 (B)
CMW 19853	Indonesia			EF686484	EF686307	EF686253		EF686352 (A)		EF686397		EF686442 (C)
CMW 19842	Indonesia	EU019893	EU019885	EF686492	EF686315	EF686261		EF686360 (A)		EF686405		EF686450 (B)
CMW 19845	Indonesia			EF686493	EF686316	EF686262	EU620605	EF686361 (C)	EU620614	EF686406	EU620622	EF686451 (A)
CMW 19891	Indonesia	EU019894	EU019886	EF686494	EF686317	EF686263		EF686362 (A)		EF686407		EF686452 (C)
CMW 19832	Indonesia			EF686496	EF686319	EF686265		EF686364 (A)		EF686409		EF686454 (C)
CMW 19831	Indonesia			EF686499	EF686322	EF686268	EU620608	EF686367 (A)	EU620609	EF686412	EU620517	EF686457 (B)
CMW 19834	Indonesia			EF686500	EF686323	EF686269		EF686368 (A)		EF686413		EF686458 (C)
CMW 13705	Thailand	EF686502	EF686271	EF686460	EF686280	EF686226		EF686325 (A)		EF686370		EF686415 (A)
CMW 13337	Thailand	EF031469	EF031481	EF031493	EF686281	EF686227		EF686326 (A)		EF686371		EF686416 (A)
CMW 13709	Thailand	EF686503	EF686272	EF686462	EF686282	EF686228		EF686327 (A)		EF686372		EF686417 (A)
CMW 13330	Thailand	EF686504	EF686273	EF686463	EF686283	EF686229		EF686328 (A)		EF686373		EF686418 (A)

Isolate no.	Location	Genbank Accession no.										
		ITS	ß-tubulin	EF-1α	CHS	ATP6	VA-1	VA-2	VA-6	VA-13	VA-15	VA-18
CMW 13710	Thailand	EF686505	EF686274	EF686464	EF686284	EF686230		EF686329 (A)		EF686374		EF686419 (A)
CMW 16136	Thailand	EF686506	EF686275	EF686465	EF686285	EF686231		EF686330 (A)		EF686375		EF686420 (A)
CMW 16124	Thailand	EF686507	EF686276	EF686466	EF686286	EF686232		EF686331 (A)		EF686376		EF686421 (A)
CMW 16123	Thailand	EF031468	EF031480	EF031492	EF686287	EF686233		EF686332 (A)		EF686377		EF686422 (A)
CMW 16120	Thailand	EF031470	EF031482	EF031494	EF686288	EF686234		EF686333 (A)		EF686378		EF686423 (A)
CMW 16138	Thailand	EF686508	EF686277	EF686469	EF686289	EF686235		EF686334 (A)		EF686379		EF686424 (A)
CMW 16137	Thailand	EF686509	EF686278	EF686470	EF686290	EF686236		EF686335 (A)		EF686380		EF686425 (A)
CMW 16126	Thailand	EF686510	EF686279	EF686471	EF686291	EF686237		EF686336 (A)		EF686381		EF686426 (A)
CMW 15089	Vietnam	EF031465	EF031477	EF031489	EF686292	EF686238		EF686337 (A)		EF686382		EF686427 (A)
CMW 15090	Vietnam	EF031466	EF031478	EF031490	EF686293	EF686239		EF686338 (A)		EF686383		EF686428 (A)
CMW 15092	Vietnam	EF031467	EF031479	EF031491	EF686294	EF686240		EF686339 (A)		EF686384		EF686429 (A)
CMW 19934	China	EU046370	EU046366	EF686472	EF686295	EF686241		EF686340 (A)		EF686413		EF686430 (B)
CMW 19914	China	EU046368	EU046364	EF686473	EF686296	EF686242		EF686341 (A)		EF686386		EF686431 (B)
CMW 19911	China	EU046369	EU046365	EF686474	EF686297	EF686243		EF686342 (A)		EF686387		EF686432 (B)
CMW 19922	China			EF686475	EF686298	EF686244		EF686343 (A)		EF686388		EF686433 (B)
CMW 19933	China	EU046371	EU046367	EF686478	EF686300	EF686247	EU620602	EF686346 (A)	EU620615	EF686391	EU620623	EF686436 (B)
CMW 19921	China			EF686501	EF686324	EF686270		EF686369 (A)		EF686414		EF686459 (B)
CMW 19909	China	EF031464	EF031476	EF031488	EF686300	EF686246		EF686345 (A)		EF686390		EF686435 (B)
CMW 19910	China	DQ632701	DQ632622	DQ632729	EF686302	EF686248		EF686347 (A)		EF686392		EF686437 (B)
CMW 19908	China	EF686511		EF686480	EF686303	EF686249	EU620607	EF686348 (A)	EU620616	EF686393	EU620624	EF686438 (B)

[1] Designation of isolates and culture collections: CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

DISCUSSION

Four nuclear gene regions, one mitochondrial gene region and four microsatellite markers (over 200 bp of sequence data) were monomorphic among 43 isolates of *K. destructans* from four countries. Two microsatellite regions showed a low level of polymorphism. Within a species there is usually a low level of nucleotide polymorphism even among 'housekeeping' genes such as ITS. For example Pegg *et al.* (2008) observed 10 ITS haplotypes among 43 isolates of *Quambalaria piterika*. Sakalidis (2005) observed 16 ITS haplotypes among 65 isolates of *Neofusicoccum parvum*. In most studies where several isolates are sequenced low levels of polymorphism (<1%) can be observed and in all our recent studies on *Kirramyces* spp. polymorphism has been observed among all species except *K. destructans* (Chapter 2). Thus to observe no polymorphisms in nine sequenced regions is very rare and this is indicative of a small founder population. The observed low level of variation in the two microsatellite regions suggests there have either been mutations from a single founder clone or that there was a low level of genetic variability in the founder population.

The leaf and shoot blight disease caused by *K. destructans* was first observed in the Lake Toba area of north Sumatra, Indonesia in 1995 (Wingfield *et al.* 1996). Thus, this is a relatively newly recognised pathogen of *Eucalyptus.* After its first appearance in North Sumatra, it was found in South Sumatra and thereafter, in Thailand in 2000, Vietnam and East Timor in 2002 (Old *et al*. 2003b) and in China in 2003 (Burgess *et al*. 2006a). Because of the intensity of production of *Eucalyptus*, it is unlikely *K. destructans* would have been in these areas for long without being detected. Thus, the sequential appearance in countries of the region over a short period of time strongly suggests anthropogenic movement probably via infected germplasm or mother plants throughout South-East Asia, linked to the rapidly growing eucalypt plantation industry. Similarly, a founder effect was observed in the genetic structure of *Mycosphaerella fijiensis* in Latin America-Caribbean region and Africa and was attributed to the movement of infected plant material by anthropogenic activities such as food wrapping with infected leaves (Rivas *et al*. 2004).

Disease caused by *K. destructans* was first observed in China 2003 in central Guangdong Province and on Hainan Dao infecting planting stock originating from Guangxi Province. In one Guangxi

nursery visited in 2004, *K. destructans* had been a serious problem since 2002. The production of cuttings from infested mother stock and poor nursery hygiene resulted in a high proportion of diseased planting stock. Infected material has been, and continues to be, widely distributed across south China, reaching isolated parts of South West Yunnan in 2003, and Fujian Province in 2006.

In China, it is most likely that the organism has been disseminated on infected cuttings from mother stock plants. It is less likely the pathogen has been distributed by spores in air moving from Vietnam into China, as the more exposed eucalypt nurseries in coastal regions of south China *e.g.* in Hainan Dao, Leizhou Peninsula and Zhangjiang were free of this pathogen in 2003/4 (B. Dell unpublished data). Seed contaminated with spores could have been inadvertently introduced into Guangxi during the China-Australia Eucalypt Afforestation Technical Cooperation Project (1981-1989) near Dongmen. However, this seems unlikely as no symptoms of *K. destructans* were reported in ACIAR field trials on plantation eucalypts in south China carried out in the 1990's. One of these trials, near Kaiping in Guandong Province, was monitored annually for two rotations from 1992 and no defoliation due to leaf disease had occurred (D. Xu pers. comm.). Thus, in China, it is likely *K. destructans* was introduced on cuttings used in the establishment of clonal nurseries.

Variation within the microsatellite loci of the *K. destructans* isolates from Indonesia indicates recent mutations or a smaller level of genetic variation in the founder population. This variability within the pathogen could lead to more pathogenic strains. We believe that while *K. destructans* has already been moved around Asia, care should now be taken not to move new genotypes throughout the region. In addition, forestry companies in the tropics and sub-tropics of South America, Africa and Australia should import germplasm, including seed from Asia, with extreme care.

CHAPTER 5

Phylogeny and movement of *Kirramyces eucalypti* across its current distribution

ABSTRACT

In eastern Australia, *Kirramyces eucalypti* is a leaf parasite of endemic *Eucalyptus* spp. but under favourable conditions can cause serious leaf blight disease mostly infecting juvenile leaves of some *Eucalyptus* spp. in plantations. Very little is known about the biology, ecology and genetics of this pathogen. The objective of this study was to determine the population diversity and gene flow of *K. eucalypti* isolates throughout Australia and New Zealand using multi-gene phylogenies and microsatellite markers. Gene genealogies and microsatellites separated *K. eucalypti* into two major populations, one from Queensland and the other from elsewhere, New South Wales, Victoria, Tasmania and New Zealand. It is likely that the Queensland population will constitute a new species; however, further research is required. Based on phylogenetic data the greatest diversity is found among isolates from New South Wales suggesting this is the origin of *K. eucalypti*. Data obtained for microsatellites were contradictory and thus considered unreliable.

INTRODUCTION

Kirramyces eucalypti is a mitosporic fungus that causes leaf blight disease. It mostly infects young leaves of some *Eucalyptus* spp. in eastern Australia and New Zealand (Figure 1). *Kirramyces eucalypti* was first described from diseased leaves of a *Eucalyptus* sp. collected from Oakleight Victoria, Australia in 1884 (Cooke 1889). The fungus was subsequently found on *E. dalrympleana* and *E. viminalis* in New South Wales (Heather 1961) and in plantations of *Eucalyptus nitens* and *E. globulus* in southern New South Wales and Tasmania (Yuan 1999). While it is a less common pathogen in mainland Australia, *K. eucalypti* was one of the three most common pathogens found on young leaves of *E. nitens* and *E. globulus* in Tasmania (Yuan 1999).

Species susceptible to *K. eucalypti* belong to the subgenus Symphyomyrtus and include; *E. camaldulensis, E. cephalocarpa*, *E. cypellocarpa*, *E. dalrympleana*, *E. dunnii*, *E. globulus*, *E. grandis*, *E. grandis* x *E. urophylla*, *E. gunnii*, *E. longirostrata*, *E. moluccana*, *E. nitens*, *E. ovata*, *E. platypus, E. parvula, E. punctata*, *E. scorparia*, *E. smithii*, *E. tereticornis* and *E. viminalis* (Carnegie 2007b). The most susceptible of these eucalypt species are *E. nitens*, *E. smithii* and clones between *E. grandis* x *E. urophylla* and *E. grandis* x *E. camaldulensis* (Carnegie, pers. comm.).

In New Zealand, *Kirramyces eucalypti* was first observed in 1981 where it was introduced with plantings of *E. nitens* from south east Australia (Gadgil & Dick 1983) and was regarded as a minor pathogen (Dick 1982). This situation changed with the establishment of short-rotation plantations of suitable eucalypt species during the 1990s, when *K. eucalypti* was found to cause complete defoliation of juvenile leaves of *E. nitens* (Hood *et al*. 2002a, 2002b). This disease outbreak happened because the *E. nitens* plantation was established in a region with warm humid weather in a coastal area, a climate considered favourable to *K. eucalypti* (Ridly 2004). In contrast, in Australia, the impact of *K. eucalypti* was considered minimal and was reported only once causing extensive damage on *E. dalrympleana* and *E. viminalis* in New South Wales (Heather 1961). However, during forest health surveys between 1996 and 2005 in New South Wales, *K. eucalypti* was found to cause significant and repeated damage to plantations of *E. nitens* and hybrids of *E. nitens* x *E. camaldulensis* in the Dorrigo Plateau (Carnegie 2007a). The affected plantations of *E. nitens* recovered poorly from damage and thus were susceptible

to other diseases such as the stem fungal pathogen, *Holocryphia eucalypti* that resulted in top-death and tree mortality (Carnegie 2007a).

Kirramyces eucalypti was found first in Victoria on a native *Eucalyptus* sp. (Cooke 1889) but its origin and pathway of movement is unknown. The aim of this study was to determine the origin and movement of *K. eucalypti* throughout its current distribution in Australia and New Zealand. In Chapter two, four gene regions for *K. eucalypti* were sequenced and variation between isolates was observed. Thus, in the present study three gene regions, one mitochondrial and two nuclear were sequenced and analysed. In addition, microsatellite markers were developed and tested on isolates collected throughout Australia and New Zealand.

Figure 1 Damage of a juvenile leaf of a *Eucalyptus* sp. caused by *Kirramyces eucalypti* in Queensland.

MATERIALS AND METHODS

Fungal isolates

Kirramyces eucalypti isolates were collected from several geographical regions where it is known to occur these included New South Wales (NSW), south Queensland (S-QLD), central Queensland (C-QLD), far north Queensland (FNQ), Victoria (VIC), Tasmania (TAS) and New Zealand (NZ) (Figure 2). *Kirramyces eucalypti* was isolated under a dissecting microscope as described in Chapter 2.

Cultures were maintained at 20ºC on 2% malt extract agar (MEA). All isolates are maintained in the Murdoch University culture collection (MUCC) or in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Fifty-seven representative isolates from a range of plantations and hosts through Australia were used; 16 from northern NSW, 18 from three regions in QLD (four from FNQ, seven from C-QLD, eight from S-QLD), 12 from TAS, three from VIC and eight from NZ (Table 1).

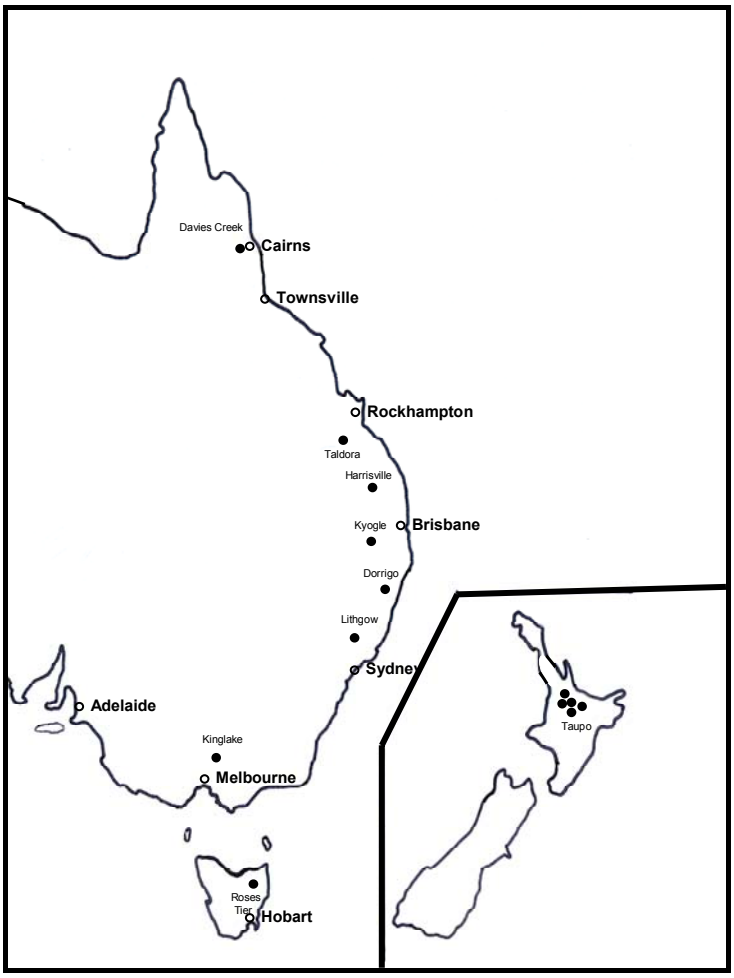


Figure 2 Map showing locations of the sampled isolates of *Kirramyces eucalypti*.

Table 1. *Kirramyces eucalypti* isolates considered in this study.

Culture no.[1]	Host	Location	Collector	GenBank accession no.			
				ATP-6	EF-1α	ß-tubulin	VA24
CMW 19455	*Eucalyptus nitens*	Coxs, New Zealand	M Dick	EU101515	EU101628	EU101571	EU101646
CMW 19461	*E. nitens*	Sun Valley, New Zealand	M Dick	EU101470	EU101583	EU101527	EU101647
CMW 19463	*E. nitens*	Sun Valley, New Zealand	M Dick	EU101471	EU101584	EU101528	EU101651
CMW 19453	*E. nitens*	Settlement Rd, New Zealand	M Dick	EU101472	EU101585	EU101529	EU101652
CMW 19456	*E. nitens*	Douthetts, New Zealand	M Dick	EU101474	EU101587	EU101531	EU101662
CMW 19462	*E. nitens*	Sun Valley, New Zealand	M Dick	EU101473	EU101586	EU101530	EU101653
CMW 19464	*E. nitens*	Sun Valley, New Zealand	M Dick	EU101475	EU101588	EU101532	EU101654
CMW 19470	*E. nitens*	Kawerau, New Zealand	M Dick	EU101476	EU101589	EU101533	EU101655
MUCC 598	*E. globulus x E. camaldulensis*	Harrisville,S-QLD, Australia	AJ Carnegie	EU101479	EU101592	EU101536	EU101685
MUCC 599	*E. globulus x E .camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	EU101480	EU101593	EU101537	EU101686
MUCC 600	*E. globulus x E .camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	EU101481	EU101594	EU101538	EU101687
MUCC 601	*E. globulus x E .camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	EU101482	EU101595	EU101539	EU101688
MUCC 602	*E. globulus x E. camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	EU101483	EU101596	EU101540	EU101692
MUCC 603	*E. globulus x E .camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	EU101484	EU101597	EU101541	EU101690
MUCC 604	*E. globulus x E. camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	EU101502	EU101615	EU101558	EU101689
MUCC 605	*E. globulus x E.. camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	EU101503	EU101616	EU101559	EU101691
MUCC 607	*E. grandis x E. camaldulensis*	Taldora, C-QLD, Australia	G. Pegg	EU101485	EU101598	EU101542	EU101693
MUCC 608	*E. grandis x E. camaldulensis*	Taldora, C-QLD, Australia	G. Pegg	EU101504	EU101617	EU101560	EU101684
MUCC 609	*E. grandis x E. camaldulensis*	Taldora, C-QLD, Australia	G. Pegg	EU101505	EU101618	EU101561	EU101696
MUCC 610	*E. grandis x E. camaldulensis*	Taldora, C-QLD, Australia	G. Pegg	EU101486	EU101599	EU101543	EU101694
MUCC 611	*E. grandis x E. camaldulensis*	Taldora, C-QLD, Australia	G. Pegg	EU101487	EU101600	EU101544	EU101681
MUCC 612	*E. grandis x E. camaldulensis*	Taldora, C-QLD, Australia	G. Pegg	EU101488	EU101601	EU101545	EU101695
MUCC 613	*Eucalyptus* sp.	Davies Creek, FNQ, Australia	TI Burgess	EU101498	EU101611	EU101554	EU101678
MUCC 614	*Eucalyptus* sp.	Davies Creek, FNQ, Australia	TI Burgess	EU101499	EU101612	EU101555	EU101677
MUCC 615	*Eucalyptus* sp.	Davies Creek, FNQ, Australia	TI Burgess	EU101500	EU101613	EU101556	EU101683
MUCC 606	*E. grandis x E. camaldulensis*	Mackay, FNQ, Australia	G Pegg	EU101516	EU101629	EU101572	EU101682
MUCC 616	*Eucalyptus* sp.	Lithgow, NSW, Australia	AJ Carnegie	EU101496	EU101609	EU101552	EU101669
MUCC 617	*Eucalyptus* sp.	Lithgow, NSW, Australia	AJ Carnegie	EU101497	EU101610	EU101553	EU101679
MUCC 618	*Eucalyptus* sp.	Lithgow, NSW, Australia	AJ Carnegie	EU101514	EU101627	EU101570	EU101648

Culture no.[1]	Host	Location	Collector	GenBank accession no.			
				ATP-6	EF-1α	ß-tubulin	VA24
MUCC 619	*E. nitens*	Dorrigo, NSW, Australia	AJ Carnegie	EU101506	EU101619	EU101562	EU101670
MUCC 620	*E. nitens*	Dorrigo, NSW, Australia	AJ Carnegie	EU101507	EU101620	EU101563	EU101671
MUCC 621	*E. nitens*	Dorrigo, NSW, Australia	AJ Carnegie	EU101508	EU101621	EU101564	EU101672
MUCC 622	*E. nitens*	Dorrigo, NSW, Australia	AJ Carnegie	EU101509	EU101622	EU101565	EU101674
MUCC 623	*E. nitens*	Dorrigo, NSW, Australia	AJ Carnegie	EU101510	EU101623	EU101566	EU101673
MUCC 624	*E. nitens*	Dorrigo, NSW, Australia	AJ Carnegie	EU101511	EU101624	EU101567	EU101675
MUCC 625	*E. nitens*	Dorrigo, NSW, Australia	AJ Carnegie	EU101512	EU101625	EU101568	EU101665
MUCC 626	*E. grandis x tereticornis*	Kyogle, NSW, Australia	AJ Carnegie	EU101489	EU101602	EU101546	EU101666
MUCC627	*E. grandis x tereticornis*	Kyogle, NSW, Australia	AJ Carnegie	EU101490	EU101603	EU101547	EU101667
MUCC 628	*E. grandis x tereticornis*	Kyogle, NSW, Australia	AJ Carnegie	EU101491	EU101604	EU101548	EU101668
MUCC 629	*E. grandis x tereticornis*	Kyogle, NSW, Australia	AJ Carnegie	EU101492	EU101605	EU101549	EU101643
MUCC 630	*E. grandis x tereticornis*	Kyogle, NSW, Australia	AJ Carnegie	EU101493	EU101606	EU101550	EU101680
MUCC 631	*E. grandis x tereticornis*	Kyogle, NSW, Australia	AJ Carnegie	EU101513	EU101626	EU101569	EU101676
MUCC 433	*E. nitens*	Kinglake, VIC, Australia	PA Barber	EU101494	DQ632726	DQ632631	EU101663
MUCC 633	*E. nitens*	Kinglake, VIC, Australia	PA Barber	EU101495	EU101608	EU101551	EU101664
MUCC 634	*E. nitens*	Kinglake, VIC, Australia	PA Barber	EU101478	EU101591	EU101535	EU101642
MUCC 635	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101501	EU101614	EU101557	EU101644
MUCC 636	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101477	EU101590	EU101534	EU101641
MUCC 637	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101517	EU101630	EU101573	EU101649
MUCC 638	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101518	EU101631	EU101574	EU101640
MUCC 639	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101519	EU101632	EU101575	EU101650
MUCC 640	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101525	EU101638	EU101581	EU101658
MUCC 641	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101520	EU101633	EU101576	EU101645
MUCC 642	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101521	EU101634	EU101577	EU101659
MUCC 643	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101522	EU101635	EU101578	EU101656
MUCC 644	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101526	EU101639	EU101582	EU101661
MUCC 645	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101523	EU101636	EU101579	EU101657
MUCC 646	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	EU101524	EU101637	EU101580	EU101660

Designation of isolates and culture collections: CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa. MUCC= Murdoch University Culture Collection, Perth, Western Australia.

DNA extraction

The isolates were grown on 2% MEA at 20°C for four weeks and the mycelium was harvested and placed in 1.5 ml sterile Eppendorf® tubes. The harvested mycelium was then frozen in liquid nitrogen, ground to a fine powder and genomic DNA extracted as described previously in Chapter 2.

Development of microsatellite markers

Microsatellite markers were developed as described in Chapter 5. To test for polymorphisms, four isolates representing the geographical range from which it has been isolated were chosen (MUCC631, New South Wales; MUCC599, Queensland; MUCC433, Victoria and CMW 19461, New Zealand). PCR and sequencing was performed as described below.

PCR amplification and sequencing

This study included the complete amplification of the mitochondrial ATPase protein gene (ATP-6), part of the β-tubulin gene region (βt), part of the elongation factor 1α gene (EF-1α), and one microsatellite locus (VA-24). Primers used for amplification of these regions are listed in Table 2. The PCR reaction mixture (25 µl), and visualisation of products were as described previously (Chapter 3). PCR conditions were as follows; ATP-6 and the microsatellite region were amplified under the following conditions; 10 cycles of 30 s at 94° C, 45 s at 37°C, 45 s at 65° C, followed by 30 cycles of 30 s at 94° C, 45 s at 45° C, 1 min at 65° C, and a final elongation step of 10 min at 68° C, the latter increasing by 3 s on every cycle. EF-1α was amplified as follows; initial denaturation of 7 min at 94 ° C, followed by 35 cycles of 1 min at 94° C, 1 min at 45° C, 2 min at 72° C and final elongation step of 10 min at 72° C. For failed amplifications, the Mg concentration was increased to 4 mM, and primer concentration to 0.9 *p*mol and the following PCR conditions were used; 7 min at 94°C, followed by 35 cycles of 1 min at 94° C, 1 min at 45° C, 2 min at 72° C and final elongation step of 10 min at 72° C. The PCR products were purified with Ultrabind®DNA purification kit (MO BIO Laboratories, Solana Beach, CA) following the manufacturer's instructions. Amplicons were sequenced as described previously (Burgess *et al.* 2005).

Table 2. Primer sets and annealing temperature used to amplify *Kirramyces eucalypti.*

Region	Oligos	Oligo Sequence (5'-3')	Amplicon size (bp)	AT (ºC)	Reference
ATP6	ATP6-1 ATP6-2	ATTAATTSWCCWTTAGAWCAATT TAATTCTANWGCATCTTTAATRTA	600	45	(Kretzer & Bruns 1999)
β-tubulin	βt2a βt2b	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	680	45-58	(Glass & Donaldson 1995)
EF-1α	EF1-728F EF1-986R	CATCGAGAAGTTCGAGAAGG TACTTGAAGGAACCCTTACC	350	45-55	(Carbone & Kohn 1999)
VA-24	VA24F VA24R	GTAATCGAACTGCCAAGGACG CTCACCTGACTGTACTAAGAG	160	37-45	This study

Base codes: R (AG), N (AGCT), S (GC), W (AT)

Haplotype network estimation

Haplotype networks were generated using the statistical parsimony method in the TCS v. 1.21 software programme (Clement *et al*. 2000). The program collapses DNA sequences into haplotypes and calculates the frequencies of haplotypes in the sample which are used to estimate haplotype out-group probabilities, that correlate with haplotype age (Donnelly & Tavare 1986; Castelloe & Templeton 1994), and then calculates an absolute distance matrix from which it estimates phylogenetic networks using a probability of parsimony, until the probability exceeds 0.95 (Templeton *et al*. 1992). The analysis was performed on the combined dataset of the ATPase, βt and EF-1α sequences.

Phylogenetic analysis

The phylogeny of the *K. eucalypti* population was estimated using a combination of parsimony and maximum likelihood methods. For each locus, DNA sequence data were assembled using Sequence Navigator version 1.01 (Perkin Elmer) and aligned in Clustal X (Thompson *et al.* 1997) and manual adjustments were made visually where necessary. All sequences derived in this study were deposited in GenBank and accession numbers are shown in Table 1.

The initial analysis was performed on each dataset alone (data not shown) and subsequent analyses were performed on a combined dataset of the ATP-6, βt and EF-1α sequences, after a partition

homogeneity test (PHT) had been performed in phylogenetic analysis using parsimony (PAUP) version 4.0b10 (Swofford 2003) to determine whether sequence data from the four separate gene regions were statistically congruent (Farris *et al.* 1995; Huelsenbeck *et al.* 1996). Parsimony analysis with heuristic search was performed in PAUP with random stepwise addition in 100 replicates with the tree bisection-reconnection branch-swapping option and the steepest-descent option off. All ambiguous and parsimony-uninformative characters were excluded; gaps were treated as a fifth character. MaxTrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees saved. Estimated levels of homoplasy and phylogenetic signal; tree length (TL), consistency index (CI) and retention index (RI) were determined (Hillis & Huelsenbeck 1992). Characters were unweighted and unordered branch and branch node support was determined using 1000 bootstrap replicates with equal probability (Felsenstein 1985). Trees were unrooted and four isolates of the closely related species *K. destructans*.

Bayesian analysis was conducted on the same aligned and combined dataset as that used in the distance analysis. First, MrModeltest v2.2 (Nylander 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v3.1 (Ronquist & Huelsenbeck 2003) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Two independent runs of Markov Chain Monte Carlo (MCMC) using 4 chains were run over 10 000 000 generations. Trees were saved each 10 000 generations, resulting in 10 001 trees. Burn-in was set at 100 001 generations (*i.e.* 101 trees), well after the likelihood values converged to stationary, leaving 9900 trees from which the consensus trees and posterior probabilities were calculated.

RESULTS

Haplotype network

There were 12 fixed polymorphic sites among the *K. eucalypti* population across the three sequenced gene regions (Table 3). One polymorphic site was detected in the ATP-6 region, seven in the β-tubulin region and four in the EF-1α region. A haplotype network constructed in TCS software resulted in 11 haplotypes among the *K. eucalypti* isolates (Figure 3). Haplotype A contained 16 isolates from QLD (three from FNQ, seven from S-QLD and six from C-QLD); Haplotype B contained one isolate from C-QLD; Haplotype C contained one isolate from S-QLD; Haplotypes D, E and F each contained one isolate from NSW; Haplotype G contained 26 isolates, six from NZ, 10 from NSW and 10 from TAS; Haplotype H contained six isolates, one isolate from NZ, two from NSW and three from VIC; Haplotype I contained one isolate from TAS; Haplotype J contained one isolate from NZ; Haplotype K contained two isolates, one from NSW and one isolate from TAS. Isolates from Queensland shared one common haplotype, but did not share any haplotypes with isolates obtained elsewhere (Figure 3). Six different haplotypes were observed in the population from NSW, of which three were shared with isolates from other geographically locations.

Phylogenetic analysis

The multiple gene genealogies for ATP6, βt and EF-1α sequence data compared 57 isolates of *K. eucalypti* with the out-group taxon *K. destructans*. The aligned data set for the combined gene regions consisted of 1328 characters of which 21 were parsimony informative and used in the analyses. The partition homogeneity test showed no significant (P=0.112) difference between the data from different genes and the data were combined. The combined data set contained no significant (P>0.01 gl= -0.47) phylogenetic signal compared to 1000 random trees. Initial heuristic searches of unweighted characters in PAUP resulted in 91 most parsimonious trees of 31 steps (CI=0.67, RI=0.96) (TreeBASE SN3585)[1]. Bayesian analysis resulted in a tree with identical topology (Figure 4, TreeBASE SN3585).

[1] TreeBASE reviewer no. 364

Isolates resembling the closely related species *K. destructans* formed a strongly supported clade with 100% bootstrap support and a Bayesian probability of 1.00 that was distant from the *K. eucalypti* isolates. Isolates within this clade were all from Queensland. The *K. eucalypti* isolates fell into two main groups which were separated by high Bayesian probabilities (1.00) but lower bootstrap values (62-69%). One group was divided into four supported clades (clades 1-4). Clade 1 contained 27 isolates of which seven were from NZ, 10 from NSW and 10 from TAS; clade 2 contained seven isolates of which one was from NZ, two from NSW, all three isolates from VIC and one from TAS; clade 3 contained two isolates of which one was from NSW and one from TAS; clade 4 contained three isolates from NSW. The second group contained only one clade (clade 5) which contained all 18 isolates from the 3 geographic locations in QLD.

Table 3. Isolates considered in the phylogenetic study. Positions of polymorphic nucleotides from aligned sequence data of ATP-6, ß-tubulin and EF-1α gene regions showing the variation between *Kirramyces eucalypti* isolates. Only parsimony informative nucleotides are shown. For comparison purposes polymorphisms not shared with the first isolate are highlighted in grey.

				ATP	EF-1α				β-tubulin						
Culture	**Country**	**Location**	**Host**	**236**	**33**	**66**	**51**	**142**	**73**	**91**	**93**	**97**	**200**	**210**	**236**
									Clade 1						
CMW 19455	New Zealand	Coxs, Taupo District	*Eucalyptus nitens*	G	C	C	C	C	T	G	A	A	T	G	C
CMW 19461	New Zealand	Sun Valley, Taupo District	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
CMW 19463	New Zealand	Sun Valley, Taupo District	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
CMW 19453	New Zealand	Settlement Rd, Taupo District	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
CMW 19462	New Zealand	Sun Valley, Taupo District	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
CMW 19464	New Zealand	Sun Valley, Taupo District	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
CMW 19470	New Zealand	Kawerau, Taupo District	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 616	NSW, Australia	Sunny corner, Lithgow	*Eucalyptus* sp.	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 617	NSW, Australia	Sunny corner, Lithgow	*Eucalyptus* sp.	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 618	NSW, Australia	Sunny corner, Lithgow	*Eucalyptus* sp.	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 619	NSW, Australia	Dorrigo	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 620	NSW, Australia	Dorrigo	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 621	NSW, Australia	Dorrigo	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 623	NSW, Australia	Dorrigo	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 624	NSW, Australia	Dorrigo	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 627	NSW, Australia	Hills property, Kyogle	*E. grandis x tereticornis*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 628	NSW, Australia	Hills property, Kyogle	*E. grandis x tereticornis*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 636	TAS, Australia	Roses Tier	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 637	TAS, Australia	Roses Tier	*E. nitens*	G	C	C	C	C	T	G	A	A	T	G	C
MUCC 638	TAS, Australia	Roses Tier	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 639	TAS, Australia	Roses Tier	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 640	TAS, Australia	Roses Tier	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 641	TAS, Australia	Roses Tier	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 642	TAS, Australia	Roses Tier	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 643	TAS, Australia	Roses Tier	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 644	TAS, Australia	Roses Tier	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C
MUCC 646	TAS, Australia	Roses Tier	*E. nitens*	T	C	C	C	C	T	G	A	A	T	G	C

Culture	Country	Location	Host	ATP	EF-1α				β-tubulin						
				236	33	66	51	142	73	91	93	97	200	210	236
									Clade 2						
CMW 19456	New Zealand	Douthetts, Taupo District	*E. nitens*	T	C	G	C	C	T	G	A	A	T	G	C
MUCC 625	NSW, Australia	Dorrigo	*E. nitens*	T	C	G	C	C	T	G	A	A	T	G	C
MUCC 629	NSW, Australia	Hills property, Kyogle	*E. grandis x tereticornis*	T	C	G	C	C	T	G	A	A	T	G	C
MUCC 433	VIC, Australia	Kinglake	*E. nitens*	T	C	G	C	C	T	G	A	A	T	G	C
MUCC 633	VIC, Australia	Kinglake	*E. nitens*	T	C	G	C	C	T	G	A	A	T	G	C
MUCC 634	VIC, Australia	Kinglake	*E. nitens*	T	C	G	C	C	T	G	A	A	T	G	C
MUCC 635	TAS, Australia	Roses Tier	*E. nitens*	G	C	G	C	C	T	G	A	A	T	G	C
									Clade 3						
MUCC 622	NSW, Australia	Dorrigo	*E. nitens*	T	T	C	T	C	T	G	A	A	T	G	C
MUCC 645	TAS, Australia	Roses Tier	*E. nitens*	T	T	C	T	C	T	G	A	A	T	G	C
									Clade 4						
MUCC 626	NSW, Australia	Hills property, Kyogle	*E. grandis x tereticornis*	T	C	C	C	C	T	C	G	A	C	A	C
MUCC 630	NSW, Australia	Hills property, Kyogle	*E. grandis x tereticornis*	T	C	G	C	T	T	C	G	A	C	A	C
MUCC 631	NSW, Australia	Hills property, Kyogle	*E. grandis x tereticornis*	T	C	G	T	C	T	C	G	A	C	A	C
									Clade 5						
MUCC 603	S-QLD, Australia	Harrisville	*E. globulus x E. camaldulensis*	G	C	C	C	T	C	G	A	G	C	A	T
MUCC 598	S-QLD, Australia	Harrisville	*E. globulus x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 599	S-QLD, Australia	Harrisville	*E. globulus x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 600	S-QLD, Australia	Harrisville	*E. globulus x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 601	S-QLD, Australia	Harrisville	*E. globulus x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 602	S-QLD, Australia	Harrisville	*E. globulus x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 604	S-QLD, Australia	Harrisville	*E. globulus x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 605	S-QLD, Australia	Harrisville	*E. globulus x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 607	C-QLD, Australia	Taldora	*E. grandis x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 608	C-QLD, Australia	Taldora	*E. grandis x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 609	C-QLD, Australia	Taldora	*E. grandis x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 610	C-QLD, Australia	Taldora	*E. grandis x E. camaldulensis*	G	T	C	C	T	C	G	A	A	C	A	T
MUCC 611	C-QLD, Australia	Taldora	*E. grandis x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 612	C-QLD, Australia	Taldora	*E. grandis x E. camaldulensis*	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 606	C-QLD, Australia	Taldora	*E. grandis x E. camaldulensis*	T	T	C	T	C	C	G	A	G	C	A	T
MUCC 613	FNQ, Australia	Davies Creek	*Eucalyptus* sp.	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 614	FNQ, Australia	DaviesCreek	*Eucalyptus* sp.	G	T	C	T	T	C	G	A	G	C	A	T
MUCC 615	FNQ, Australia	Davies Creek	*Eucalyptus* sp.	G	T	C	T	T	C	G	A	G	C	A	T

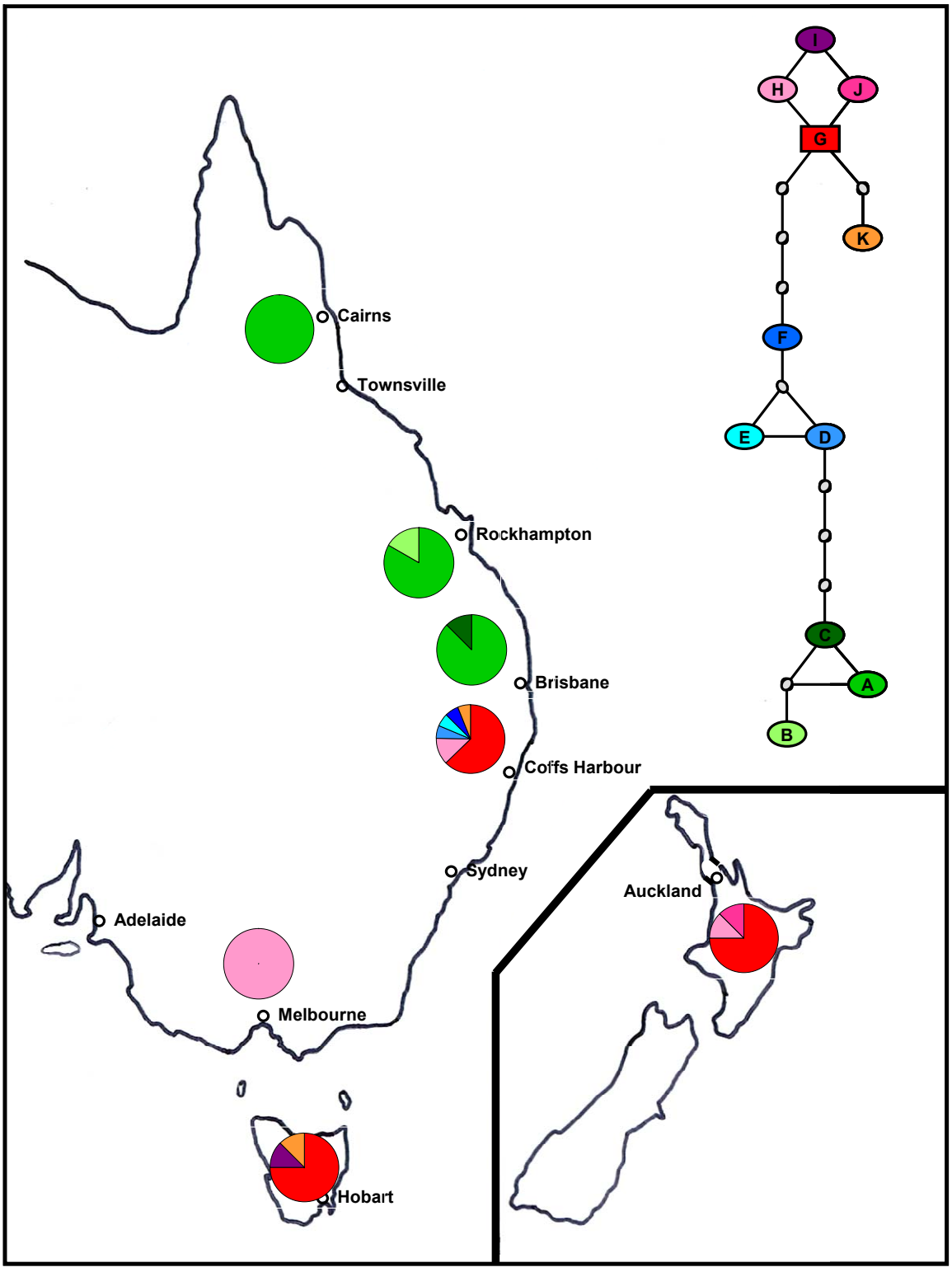


Figure 3 Distribution and proportion of the 11 detected haplotypes estimated by TCS 1.21 software. Also shown is a haplotype network, where the haplotype identity is given. The haplotypes are indicated by colours.

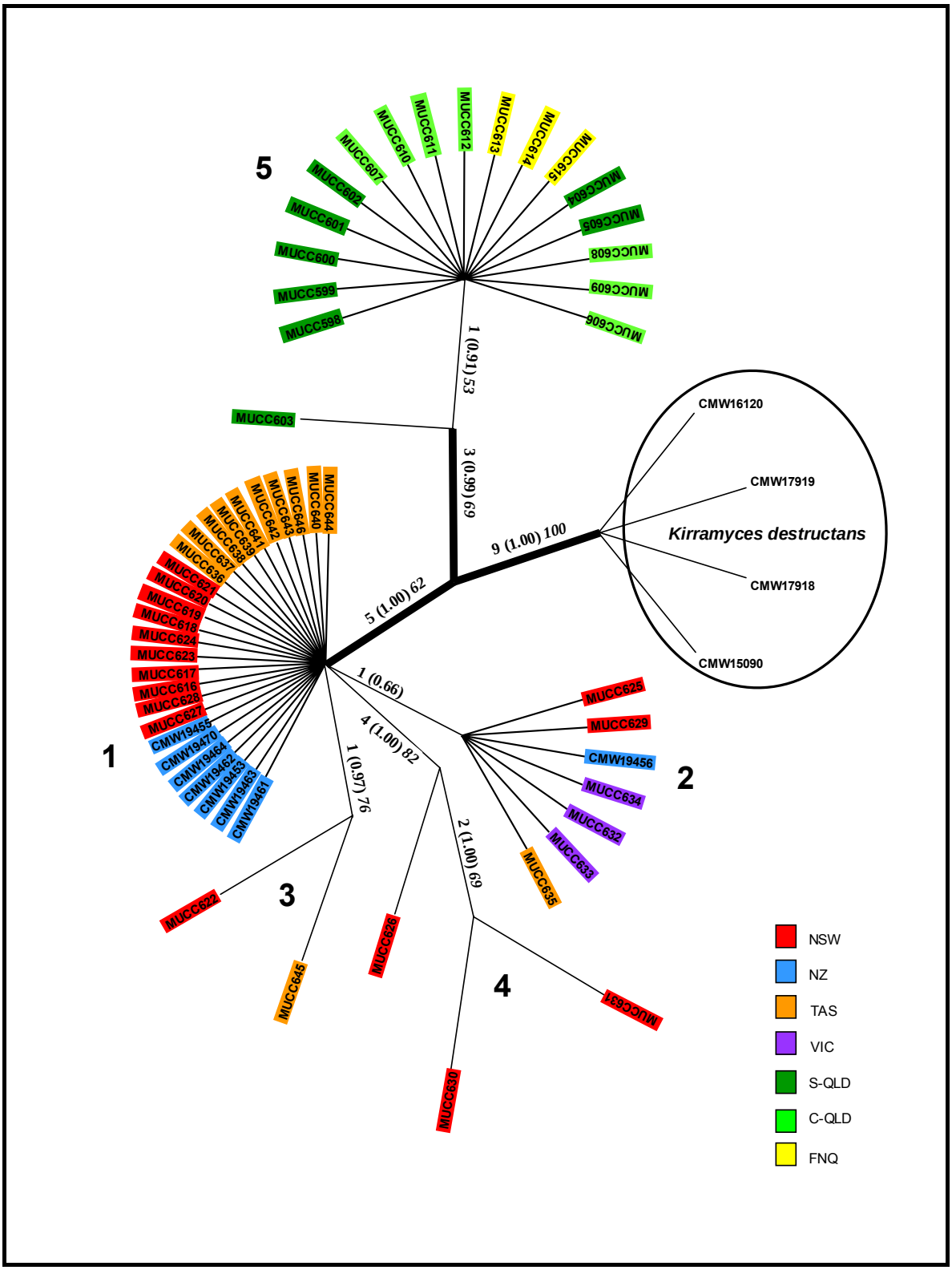


Figure 4 Consensus phylogram of 9900 trees resulting from Bayesian analysis of the combined ATP-6, βT and EF-1α sequence data for isolates of *Kirramyces eucalypti*. Branch length, posterior probabilities of the branch nodes (brackets) and bootstrap values resulting from parsimony analysis (italics) are given for each of the branches. Location of the isolates is colour coded.

Microsatellite markers

Six of the 25 primer pairs amplified a microsatellite-containing region for all tested representative isolates of *K. eucalypti,* but only one, VA-24 was polymorphic. Thus, this marker was chosen for further amplification and was sequenced for the 57 selected isolates. The marker VA-24 amplified clones containing the TG motif (Figure 5).

The microsatellite marker (VA-24) divided isolates of *K. eucalypti* into 13 haplotypes (Figure 5). Haplotypes A-D each contained one isolate and were all from TAS. Haplotype E was predominant and present in five regions and contained 32 isolates of which eight were from NZ, 13 from NSW, one from FNQ, two from VIC and eight from TAS. Haplotype F contained one isolate from FNQ and one isolate from NSW. Haplotype G contained one isolate from NSW. Haplotypes H-I each contained one isolate from C-QLD. Haplotype J contained one isolate from FNQ. Haplotype K contained one isolate from C-QLD. Haplotype L contained all eight isolates from S-QLD. Finally, haplotype M contained five isolates from C-QLD (Figure 5).

Only one haplotype (E) was observed among isolates from NZ. Five haplotypes (A-E) were observed among the isolates from TAS. Two haplotypes (B and E) were found in the population from VIC. Four haplotypes (B, E, F and G) were observed for isolates from NSW. All isolates from S-QLD were of haplotype L. Four haplotypes were observed (H, I, K and M) for isolates from C-QLD. Three haplotypes (E, F and J) were observed among isolates from FNQ (Figure 5). With the exception of haplotypes E and F found for one isolate each in FNQ and one isolate from NSW, no other haplotypes were shared between QLD and elsewhere. Based on numbers of haplotypes, the population from Tasmania was the most variable.

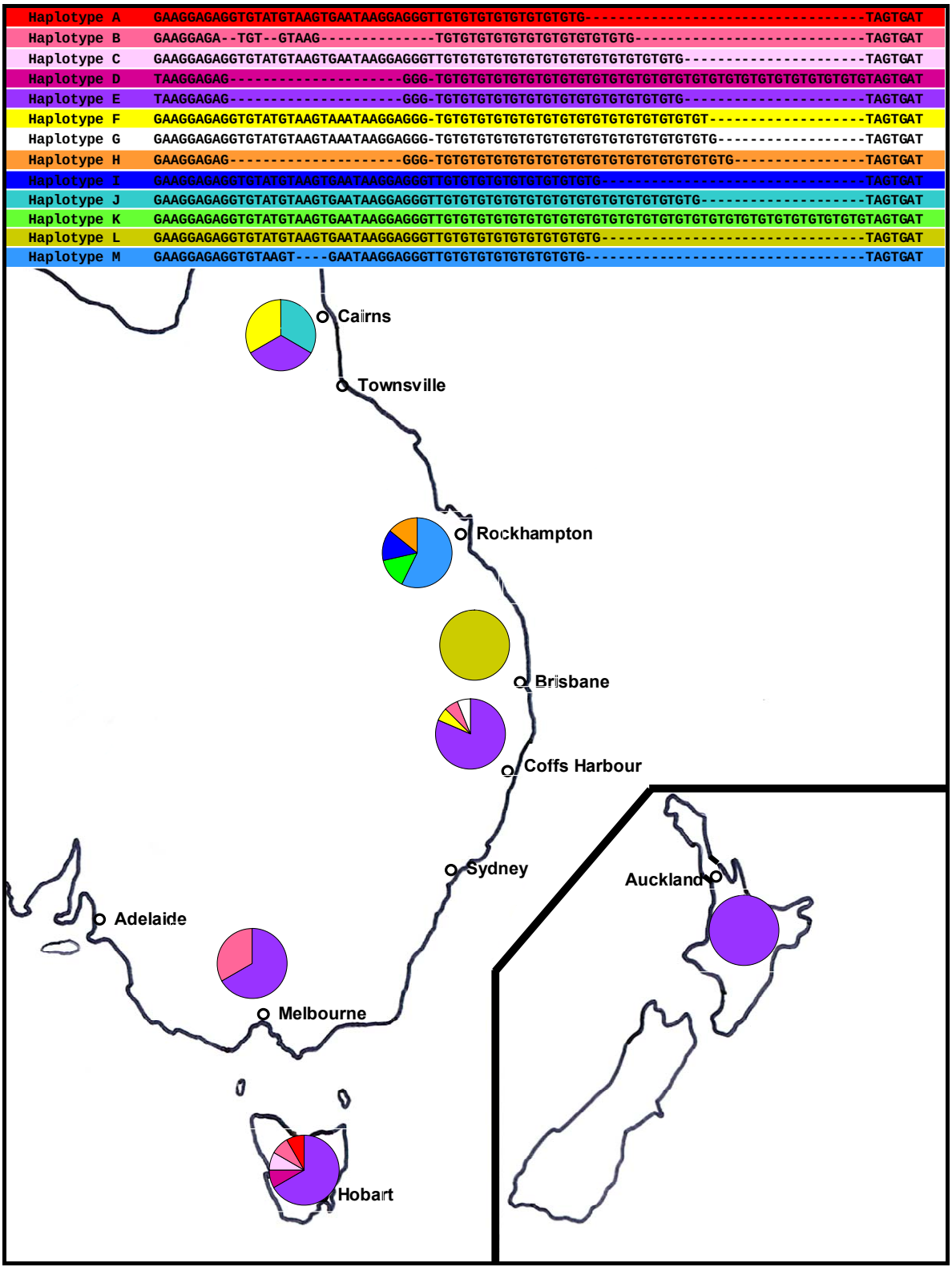


Figure 5 Distribution and proportion of the thirteen detected haplotypes obtained from sequence data of a single microsatellite locus. The haplotypes are indicated by colours and the polymorphisms within the microsatellite region which led to the different haplotypes are also shown.

DISCUSSION

The population structure of the leaf blight pathogen *K. eucalypti,* was examined using nucleotide sequence variation of three gene and one microsatellite region. Twelve polymorphic sequence sites were found in two genomic and one mitochondrial gene (1346 bp of sequence) for isolates of *Kirramyces eucalypti* from *Eucalyptus* spp. in Australia and New Zealand. The basic data set contained 11 haplotypes which collapsed into five supported clades in both parsimony and Bayesian analysis. Both phylogenetic and parsimony analysis separated isolates of *K. eucalypti* into two major clades. One clade included isolates from QLD, and another included isolates from the eastern seaboard of Australia and New Zealand. This is the first record of *K. eucalypti* for QLD. Isolates from New South Wales were further split to three sub-clades, one sub-clade also included all isolates from VIC. Six of 16 of isolates from NSW had variable DNA sequences indicating high levels of diversity in NSW. Furthermore, based on multiple gene phylogeny, there were no shared haplotypes between isolates from QLD and those from elsewhere.

Separations of isolates in multiple gene phylogeny and haplotypes based on microsatellite data were not in agreement and data from the microsatellite loci were disregarded when considering origin and movement. The sharing of haplotypes between regions in data from the multiple gene phylogeny indicate that there has been some gene flow of *K. eucalypti* both within the eastern seaboard of Australia (NSW, VIC and TAS) and between Australia and New Zealand. Based on phylogeny, the greatest diversity was found among isolates of *K. eucalypti* from northern NSW, suggesting this area could possibly be the origin of the species.

Origin and movement of K. eucalypti

Kirramyces eucalypti has been recorded in eastern Australia in New South Wales, Victoria and Tasmania and now in Queensland. It is known to have been introduced into New Zealand. The natural distribution of this pathogen in eastern Australia is unknown. *Kirramyces eucalypti* is endemic to the eastern seaboard of mainland Australia, but is it endemic to Tasmania? The highest sequence variations obtained in the present study were observed among the isolates from NSW. *Kirramyces eucalypti* isolates were collected from three geographical regions in NSW; Dorrigo, Hills Property and

Sunny Corner. Isolates from the Dorrigo plantation were more variable than isolates from Hills property, while isolates from Sunny Corner were uniform and identical with most of the isolates from NSW. The seed lot used to establish *E. nitens* at the Dorrigo plantation was a NSW provenance (Carnegie, pers. comm.), whilst the hybrids of *E. grandis* and *E. tereticornis* planted at the Hills property were from South Africa (Carnegie, pers. comm.). *Kirramyces eucalypti* has not been found in South Africa. This indicates that *K. eucalypti* must have moved from native *Eucalyptus* spp. or a nearby eucalypt plantation onto the introduced hybrids. From the data the highest diversity of *K. eucalypti* was detected in northern NSW (Dorrigo) and the presence of the same haplotypes in VIC and TAS suggests recent movement of *K. eucalypti* across eastern Australia.

Kirramyces eucalypti was first described in Victoria, but no diversity was found among the isolates collected there. If VIC was the origin of *K. eucalypti*, it would be expected to have higher genetic diversity than elsewhere. This implies that the *K. eucalypti* does not originate from VIC, but the source of seed used for the *E. nitens* trials was from VIC (Toorongo and Rubicon provenance, Barber pers. comm.) thus, there was no evidence of movement of germplasm (and potential pathogens) from another region. Comparing the results from this study with the planting history in Victoria leads to the conclusion that *K. eucalypti* is endemic to Victoria, but the number of isolates used in this study were insufficient to prove this. In the last two years, Victoria has had very dry seasons, resulting in environmental conditions that were not conducive for the collection of more isolates (D. Smith, pers. comm.).

Low sequence variation was observed among isolates from Tasmania. Ten of the 12 isolates from Tasmania were uniform and were placed in the same clade with isolates from NSW and NZ. Two isolates were variable; one was placed in the same clade as isolates from NSW and another with isolates from NSW, VIC, and NZ. Confirmation of where the source of seeds was from used to plant *E. nitens* in Tasmania could not be confirmed, but it is believed to be from the Toorongo provenance, Victoria (T. Wardlaw, pers. comm.). If the seed lot used to establish the plantations in Tasmania were from the Tooronga provenance it would be expected to have a similar sequence and the same

haplotype as the isolates from Victoria. However, there were no shared haplotypes between isolates from TAS and VIC.

Low sequence variation was observed in the New Zealand population. *Kirramyces eucalypti* isolates were collected from five different plantations of *E. nitens* in NZ. Six of eight isolates used in this study were uniform and two (Cox and Douthetts) had variable DNA sequences, but were placed in the same clade with isolates from NSW and Victoria. This low sequence variation suggests that there have been very few introductions of this fungus into New Zealand. The seed lots used to establish the *E. nitens* plantations in New Zealand is believed to be from the Toorongo provenance (Victoria) and a northern NSW provenance. With the exception of one unique haplotype found in TAS, all haplotypes in VIC, TAS and NZ are also present in NSW. This suggest historical movement of *K. eucalypti* from NSW to other regions. Evidence of seed lots used to establish trials and plantations suggest most seed has originated in VIC. Thus, either VIC haplotype first moved to NSW and was distributed elsewhere or moved directly from VIC to other regions. TAS and NZ have definitely acquired other haplotypes from NSW.

Kirramyces eucalypti isolates from Queensland were phylogenetically distinct from the isolates collected from elsewhere. The pathogen has not previously been reported in QLD. The isolates of *K. eucalypti* were collected from three locations in Queensland (far north, central and south) which are geographically distant. They were initially identified by morphology of conidia as *K. eucalypti*, however they had longer conidia, more reminiscent of *K. destructans*. When the sequence data were initially compared with previously sequenced *K. eucalypti* isolates (Chapter 2), only three base pair differences were found thus, isolates from Queensland were identified as *K. eucalypti*. However, the multi-gene phylogeny in this study revealed that there were nine polymorphic sites that separate the QLD population from the NSW population. Although the isolates from QLD were collected from three different geographic localities, the sequence variation between them was very low. Hybrids of *E. globulus* and *E. camaldulensis* used to establish the eucalypt plantations in S-QLD were of the QLD provenance, while hybrids between *E. grandis* and *E. camaldulensis* and *E. urophylla* used in C-QLD and FNQ were from South America. Although from different provenances, the damage caused by this

pathogen was severe on all hybrids in all three locations. The susceptibility of these non-endemic clones and eucalypt hybrids to *K. eucalypti* in QLD most likely reflects selection for growth and disease resistance in the absence of *K. eucalypti*. The isolates from Queensland probably represent a new cryptic species which may have evolved through selection and geographic isolation. Recombination leading to cryptic speciation has been seen in *Paracoccidioides brasiliensis*, an important human pathogen, endemic to Latin America (Restrepo 2003). Whilst considered to be a clonal species by mycological criteria, this assumption was not supported by multi gene phylogenies. As a result *Paracoccidioides brasiliensis* was divided to three distinct species (Matute *et al.* 2006). A similar situation has been shown with *Diplodia pinea*, where morphotype B was shown to have high diversity and could be further divided in relation to geographic origin (Burgess *et al*. 2001). Later, this morphotype, based on multi gene phylogeny and microsatellite loci, was described as a new species, *Diplodia scrobiculata* (de Wet *et al*. 2003). This is a similar situation to that observed with *K. eucalypti* in Australia and could result in the isolates from Queensland being described as a new species. However, additional work, including the sequencing of more gene regions, is required to resolve this relationship.

Validity of microsatellite data

High sequence variation was observed for the microsatellite locus, but this was to be expected as microsatellites are highly variable (Kimmel *et al*. 1996) with exceptionally high mutation rates (Levinson & Gutman 1987). Genetic diversity observed in the microsatellite region of *K. eucalypti* was the result of a number of variations in the tandem repeated unit (in this case TG) and indels in the flanking region.

Data derived from microsatellite alleles were in conflict with data inferred by phylogeny. Unlike sequence data, microsatellite data did not divide *K. eucalypti* isolates into groups. The most common haplotype was haplotype E that was shared between isolates from NZ, NSW, FNQ, TAS and VIC. These findings were different to the one obtained from the gene genealogies, where no shared haplotypes was found between isolates from QLD and isolates from elsewhere. Furthermore, based on microsatellite data some regions contained more haplotypes than observed by phylogeny. The most

microsatellite haplotypes was observed among the isolates from Tasmania. This would suggest that the origin of *K. eucalypti* is from Tasmania. This is contrary to the results obtained with gene genealogies, where the most gene variations were observed in the population from NSW.

Conflict between data obtained from microsatellite alleles and phylogeny was also found by studies of Sargent *et al*. (2003) in attempt to resolve the relationship with the population of *Fragaria* species. While phylogeny supported the findings from previous studies on *Fragaria*, the relationships inferred by microsatellites data were unresolved and poorly supported due to alleles showing convergence in length (Sargent *et al*. 2004). Microsatellite alleles in present study were hyper variable. Sometimes, a hyper variable locus can overestimate tree topology, such as in case of Bot*F*19 designed for *Neofusicoccum* spp. This locus was excluded from further studies (Slippers *et al*. 2004, Burgess *et al*. 2006b).

Microsatellites were useful in other studies such as those of de Wet *et al*. (2003), where both phylogeny and microsatellite data supported the placement of morphotype B of *Diplodia pinea* into the distinct taxon of *Diplodia scrobiculata*. In a similar way based on phylogeny obtained by ITS and five microsatellites markers the ectomycorrhizal fungus, *Rhizopogon vinicolor* was split into two distinct clades suggesting two biological species (Kretzer *et al*. 2003). Results in the current study were only based on one microsatellite locus; therefore, more markers need to be tested before the decision of their use in population studies of *K. eucalypti* can be made.

In conclusion, the analysis of data from the current study show that the particular microsatellite marker developed when used on its own is not a useful phylogenetic marker, although this may not be the situation if more polymorphic markers were available. Whilst multi gene genealogies suggest that isolates from QLD may represent a different species to the rest of the Australian and New Zealand isolates, microsatellites alleles are shared between the isolates from all regions. This study has shown that there are two populations of *K. eucalypti* that could led to species separation but this needs to be investigated further. The observed genetic variation in the three gene regions from this study suggests that origin of *K. eucalypti* is New South Wales.

E. nitens is a commonly used plantation species in places with a moderate climate because of its fast growth and resistance to cold. It is native to Victoria and New South Wales and usually inhabits high altitude slopes (Boland *et al*. 2006). If planted under unfavourable conditions, *E. nitens* becomes one of the most susceptible eucalyptus species to *K. eucalypti* (Hood *et al*. 2002b). Our study confirmed that *E. nitens* is the most susceptible species to *K. eucalypti* and care should be taken not to plant it in warm and humid sites. Hybrids from South Africa and South America were highly susceptible to *K. eucalypti*, and should not be planted in the sub-tropics and tropics in Australia before being tested for their susceptibility to this pathogen.

CHAPTER 6

Kirramyces viscidus sp. nov., a new eucalypt pathogen from tropical Australia closely related to the serious leaf pathogen, *Kirramyces destructans*

Published as: Andjic V, Barber PA, Carnegie AJ, Pegg GS, Hardy GEStJ, Wingfield MJ, Burgess TI, 2007. *Kirramyces viscidus* sp. nov., a new eucalypt pathogen from tropical Australia closely related to the serious leaf pathogen, *Kirramyces destructans*. *Australasian Plant Pathology,* **36:** 478-487.

Minor authors' contributions: Burgess TI and Hardy GEStJ were supervisors, Carnegie AJ and Pegg GS provided the leaf material, Barber PA and Wingfield MJ provided guidance and expertise in taxonomy.

ABSTRACT

Kirramyces destructans is a serious pathogen causing a leaf, bud and shoot blight disease of *Eucalyptus* plantations in the sub-tropics and tropics of south-east Asia. During surveillance of eucalypt taxa trials in Northern Queensland, symptoms resembling those of *K. destructans* were observed on *E. grandis* and *E. grandis* x *E. camaldulensis*. Phylogenetic and morphological studies revealed that the *Kirramyces* sp. associated with these symptoms represents a new taxon described here as *K. viscidus* sp. nov, which is closely related to *K. destructans.* Plantation assessments revealed that while *E. grandis* from the Copperload provenance, collected in northern Queensland, recovered from disease, *E. grandis* x *E. camaldulensis* hybrids from South America were highly susceptible to infection by *K. viscidus* and are not recommended for planting in northern Queensland. Preliminary results suggest the fungus probably originates from Australia. *Kirramyces viscidus* is closely related to *K. destructans,* and causes a disease with similar symptoms, suggesting that it could seriously damage Australian eucalypt plantations, especially those planted off-site.

INTRODUCTION

The eucalypt plantation industry in Australia is relatively new, with major expansion from approximately 200,000 ha to 600,000 ha occurring only in the last 10 years (National Forestry Inventory 2004). These plantations are predominantly of *Eucalyptus globulus,* situated in southern Australia and the major end- use is paper and pulp. A comparatively small eucalypt plantation industry for both timber and pulpwood is developing in the subtropical regions of northern New South Wales and southern Queensland and expanding into the tropical regions of northern Queensland (Carnegie *et al*. 2005; Dickinson *et al.* 2004). The majority of these plantations are established on previous agricultural land and do not involve the clearing of native vegetation.

Eucalypt species trials have been established throughout Australia to test the suitability of different environments for growing eucalypts. Some of these trials have been planted in the east coast tropics in a region referred to as far north Queensland (Dickinson *et al*. 2004; Lee 2007), where the climate is similar to South East Asia. The eucalypt species being tested in these trials include *E. grandis*, *E. camaldulensis* and hybrids between these two species, which are commonly used in tropical plantation forestry world-wide (Turnbull 2000).

Several eucalypt diseases have emerged in South East Asia that are unknown in Australia (Barber 2004; Burgess *et al*. 2006a; Wingfield *et al*. 1996). These diseases threaten the biosecurity of Australia's eucalypts. Under an existing project, eucalypt taxa trials in Northern Australia are currently being monitored for incursions of pathogens from Australia's northern neighbours such as Indonesia and Papua New Guinea (Burgess, pers. comm.).

Kirramyces J. Walker *et al*. species are anamorphs of *Mycosphaerella* Johanson. Species such as *K. zuluensis* (M.J. Wingf., Crous & T.A. Cout.) Andjic & M.J. Wingf., *K. destructans* (M.J. Wingf. & Crous), *K. eucalypti* (Cooke & Massee) J. Walker, B. Sutton & Pascoe and *K. epicoccoides* (Cooke & Massee) J. Walker, B. Sutton & Pascoe, are common and important pathogens of eucalypts (Wingfield *et al*. 1997; Park *et al*. 2000; Carnegie 2007b). *Kirramyces zuluensis* and *K. destructans* are unknown in Australia. *Kirramyces zuluensis* causes a serious stem canker disease on *Eucalyptus* species in

subtropical areas of South Africa (Wingfield *et al.* 1997), Hawaii (Cortinas *et al*. 2004), Ethiopia and Uganda (Gezahgne *et al.* 2005), Argentina and Vietnam (Gezahgne *et al*. 2003), China (Cortinas *et al*. 2006b) and Uruguay (M.J. Wingfield, unpublished). *Kirramyces destructans* is an aggressive, destructive pathogen causing distortion of infected leaves and blight of young leaves, buds and shoots (Wingfield *et al*. 1996). This pathogen was first discovered in Indonesia in 1996 and has subsequently been detected in Thailand, China, Vietnam and Timor (Old *et al*. 2003a; Old *et al*. 2003b; Wingfield *et al.* 1996; Burgess *et al*. 2006a). *Kirramyces eucalypti* and *K. epicoccoides* are endemic to Australia and outbreaks have been noted in off-site plantations in the sub-tropics. For example, in northern New South Wales, *K. eucalypti* can cause severe damage to *E. nitens* plantations and *K. epicoccoides* can cause severe, repeated defoliation in *E. grandis* and *E. grandis x E. camaldulensis* plantations (Carnegie 2007b). *Kirramyces eucalypti* has also been introduced to New Zealand where it has resulted in the complete defoliation of juvenile leaves of *E. nitens* in New Zealand (Dick 1982). *Kirramyces epicoccoides* is known from many countries (Park *et al*. 2000; Sankaran *et al*. 1995) and generally causes disease on older leaves, although leaf blights have been observed in the tropics (Dell, pers. comm.).

During monitoring of eucalypt species trials in far north Queensland in July 2005, leaves exhibiting symptoms similar to those observed for *K. destructans* were observed (Burgess, unpub. data). Samples were collected across several sites and preliminary examination revealed a fungus with a conidial morphology similar to that of *K. destructans*. This study describes the fungus as a new *Kirramyces* species from northern Queensland and discusses its relationship to *K. destructions*. The origin of these species and the necessity for conducting surveillance and risk assessment are also considered.

MATERIALS AND METHODS

Collection

Eucalyptus leaves with symptoms resembling those of *K. destructans* were collected from a eucalypt species trial site situated 3 km north-west of Mareeba, near Cairns in far north Queensland, Australia. The trial was established in August 2004 to evaluate hardwood species grown using effluent irrigation in the dry tropics. Species planted in the trial included *Corymbia* spp., *E. pellita, E. grandis* and hybrids of *E. grandis* x *E. camaldulensis* from South America. Collected leaves were wrapped in paper tissues, placed in paper bags, packed in tight- fitting plastic bags from which the air had been displaced to form a vacuum and stored in a fridge prior to the isolation of fungi.

Fungal isolates

Leaves were examined under a dissecting microscope, and conidia observed oozing from single pycnidia, were collected at the tip of a sterile needle. The conidia were transferred to malt extract (20 g l^{-1}) agar (MEA) containing Streptomycin 150µg/ml (Sigma-Aldrich, Australia) in a single spot and allowed to hydrate for 2-4 hrs. Under the dissecting microscope, conidia were then streaked across the surface of the agar using a sterile needle and single conidia were picked off the agar and transferred to new MEA plates. Cultures were grown at 20° C in the dark for two weeks and then transferred to fresh MEA plates. Cultures were maintained on 2% MEA in tubes at 20º C. Morphological characteristics of the isolates from this study were compared to those of other closely related species (Table 1). All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa or at Murdoch University (MUCC). Ex-type cultures have been deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht. The holotype has been lodged in the herbarium of the Department of Primary Industries, Brisbane (BRIP). Isotype and paratype material is available from the Murdoch University herbarium (MURU).

Table 1. *Kirramyces* species and isolates considered in the phylogenetic study.

Culture no.[1]	Teleomorph	Anamorph	Host	Location	Collector	GenBank accession no.		
						ITS	ß-tubulin	EF-1α
MUCC 452, CBS 121156		*Kirramyces viscidus*	*E. grandis*	Mareeba, Australia	TI Burgess	**EF031471**	**EF031483**	**EF031495**
MUCC 453, CBS 121157		*K. viscidus*	*E. grandis*	Mareeba, Australia	TI Burgess	**EF031472**	**EF031484**	**EF031496**
MUCC 454		*K. viscidus*	*E. grandis*	Mareeba, Australia	TI Burgess	**EF031473**	**EF031485**	**EF031497**
MUCC 45		*K. viscidus*	*E. grandis*	Mareeba, Australia	TI Burgess	**EF031474**	**EF031486**	**EF031498**
MUCC 456, CBS 121155		*K. viscidus*	*E. grandis*	Mareeba, Australia	TI Burgess	**EF031475**	**EF031487**	**EF031499**
MUCC 468		*K. viscidus*	*E. grandis x E. camaldulensis*	Mareeba, Australia	TI Burgess	**EF527436**	**EF527430**	**EF527433**
MUCC 469		*K. viscidus*	*E. grandis x E. camaldulensis*	Mareeba, Australia	TI Burgess	**EF527438**	**EF527432**	**EF527435**
MUCC 467		*K. viscidus*	*E. grandis x E. camaldulensis*	Mareeba, Australia	TI Burgess	**EF527437**	**EF527431**	**EF527434**
CMW 22553		*K. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	DQ632667	DQ632625	DQ632732
CMW 17918		*K. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	DQ632666	DQ632624	DQ632731
CMW 19832		*K. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	DQ632665	DQ632623	DQ632730
CMW 17919		*K. destructans*	*E. urophylla*	Guangzhou, China	TI Burgess	DQ632701	DQ632622	DQ632729
CMW 19909		*K. destructans*	*E. urophylla*	Guangzhou, China	TI Burgess	EF031464	**EF031476**	**EF031488**
CMW 15089		*K. destructans*	*E. camaldulensis*	Vietnam	TI Burgess	EF031465	**EF031477**	**EF031489**
CMW 15090		*K. destructans*	*E. camaldulensis*	Vietnam	TI Burgess	EF031466	**EF031478**	**EF031490**
CMW 15092		*K. destructans*	*E. camaldulensis*	Vietnam	TI Burgess	EF031467	**EF031479**	**EF031491**
CMW 16123		*K. destructans*	*E. camaldulensis*	Thailand	MJ Wingfield	EF031468	**EF031480**	**EF031492**
CMW 13337		*K. destructans*	*E. camaldulensis*	Thailand	MJ Wingfield	EF031469	**EF031481**	**EF031493**
CMW 16120		*K. destructans*	*E. camaldulensis*	Thailand	MJ Wingfield	EF031470	**EF031482**	**EF031494**
CMW 17915		*K. eucalypti*	*E. nitens*	Victoria, Australia	PA Barber	DQ632664	DQ632626	DQ632727
CMW 17917		*K. eucalypti*	*E. grandis x E. tereticornis*	New South Wales	AJ Carnegie	DQ632711	DQ632630	DQ632725
CMW 17916		*K. eucalypti*	*E. grandis x E. camaldulensis*	Queensland	AJ Carnegie	DQ632659	DQ632628	DQ632722
CMW 11687		*K. eucalypti*	*E. nitens*	New Zealand	M Dick	DQ240001	DS890168	DQ235115
MUCC 538	*M. suttonii*	*K. epicoccoides*	*E. globulus*	Western Australia	S Jackson	DQ632702	DQ632619	DQ632716
MUCC 425	*M. suttonii*	*K. epicoccoides*	*E. grandis*	New South Wales	TI Burgess	DQ632655	DQ632613	DQ632713
CMW 22484	*M. suttonii*	*K. epicoccoides*	*E. urophylla*	China	TI Burgess	DQ632705	DQ632616	DQ632714
SA12	*M. suttonii*	*K. epicoccoides*	*E. fragrata*	South Africa	MN Cortinas	DQ632657	DQ632614	DQ632718
CBS 113313, CMW 14457	*M. toledana*	*K. toledana*	*E. globulus*	Spain	PW Crous	AY725581	DQ658235	DQ235120

Culture no.[1]	Teleomorph	Anamorph	Host	Location	Collector	GenBank accession no.		
						ITS	ß-tubulin	EF-1α
CMW 11560	*M. nubilosa*		*E. globulus*	Tasmania	A Milgate	DQ658232	DQ658236	DQ240176
CMW 3279	*M. cryptica*	*K. nubilosum*	*E. globulus*	Australia	AJ Carnegie	AY309623	DQ658234	DQ235119
CBS 117262, CMW 7449		*K. zuluensis*	*E. grandis*	South Africa	L Van Zyl	DQ240021	DQ240102	DQ240155
CBS 113399, CMW 13328		*K. zuluensis*	*E. grandis*	South Africa	L Van Zyl	DQ240018	DQ658233	DQ240172
CBS 110499, CMW 13704	*M. molleriana*	*K. molleriana*	*E. globulus*	Western Australia	A Maxwell	AY150675	DQ240116	DQ240169
CMW 4940	*M. molleriana*	*K. molleriana*	*Eucalyptus* sp.	Portugal	MJ Wingfield	DQ239969	DQ240115	DQ240168
CMW 11588	*M. molleriana*	*K. molleriana*	*E. globulus*	Tasmania	A Milgate	DQ239968	DQ240114	DQ240167
CMW 7773	*Neofusicoccum ribis*		*Ribes* sp.	New York, USA	B Slippers	AY236936	AY808170	AY236878

[1] Designation of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; MUCC = Murdoch University culture collection, Australia

[2] Sequences in bold were obtained during this study

DNA Extraction and PCR amplification

The isolates were grown on 2% MEA at 20° C for 4 weeks and the mycelium was harvested and placed in a 1.5ml sterile Eppendorf® tube. Harvested mycelium was frozen in liquid nitrogen, ground to a fine powder and genomic DNA was extracted as described previously in Chapter 2. The region spanning the second internal transcribed spacer and part of 5.8S region of the rDNA was amplified using the primers ITS-3 (5' GTA TCG ATG AAG AAC GCA GC 3') and ITS-4 (5'TCC TCC GCT TAT TGA TAT GC 3') White *et al*. (1990). Part of the β-tubulin (βt) gene region was amplified with the primers βt2a (5'GGT AAC CAA ATC GGT GCT GCT TTC 3') and βt2b (5'ACC CTC AGT GTA GTG ACC CTT GGC 3') Glass & Donaldson (1995), part of translation elongation factor-1α(EF-1α) gene with the primers EF1-728 (5'CAT CGA GAA GTT CGA GAA GG 3') and EF1-986R (5' TAC TTG AAG GAA CCC TTA CC 3') Carbone & Kohn (1999). The PCR reaction mixture, PCR conditions, the clean- up of products and sequencing were as described previously in Chapter 2.

Phylogenetic analysis

In order to compare *Kirramyces* isolates used in this study with other closely related species, additional sequences were obtained from GenBank (Table 1). Sequence data were assembled using Sequence Navigator version 1.01 (Perkin Elmer) and aligned in Clustal X (Thompson *et al.* 1997). Manual adjustments were made visually by inserting gaps where necessary. All sequences derived in this study were deposited in GenBank and accession numbers are shown in Table 1.

Analyses were performed on individual data sets in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2003). The most parsimonious trees were obtained using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option, off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis & Huelsenbeck 1992).

Branch and branch node support was determined using 1000 bootstrap replicates (Felsenstein 1985). Trees were rooted to *Neofusicoccum ribis*, which was treated as the outgroup taxon.

Bayesian analysis was conducted on the same aligned and combined dataset as the one used in the distance analysis. First, MrModeltest v2.2 (Nylander 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v3.1 (Ronquist & Huelsenbeck 2003) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Two independent runs of Markov Chain Monte Carlo (MCMC) using 4 chains were run over 1 000 000 generations. Trees were saved each 1 000 generations, resulting in 10 001 trees. Burn-in was set at 50 001 generations (*i.e*. 51 trees), well after the likelihood values converged to the stationary, leaving 9950 trees from which the consensus trees and posterior probabilities were calculated.

Figure 1. Heavily defoliated 15-month-old *Eucalyptus grandis* x *E. camaldulensis* at Mareeba, Atherton Tableland. A year earlier the trees had full foliage and early symptoms of infection with *Kirramyces viscidus*.

Morphological characterisation

In order to determine the taxonomic position of the *Kirramyces* species considered in this study, plugs (2mm diam.) were cut from actively growing cultures and placed at the centres of Petri dishes (55 mm) containing one of three different nutrient media. Three replicates of each isolate (5 isolates in total) were grown on 2% Malt Extract Agar (MEA), oatmeal agar (OMA) and sterilised eucalypt leaves placed on the surface of tap water agar (TWA) at 20° C and 28° C in the dark. After 30 days, cultures were assessed for growth and photographed. Squash mounts of fruiting structures were prepared on slides in lacto-glycerol (1:1:1 volume of lactic acid, glycerol and water) and observed at 1000 x magnification with an Olympus BH2 light microscope. The growth of cultures was determined by taking two measurements of colony diameter perpendicular to each other. Measurements of relevant taxonomic features used to distinguish between currently described *Kirramyces* spp. were made. Thus, each isolate was assessed for conidial size, shape, pigmentation and number of septa. Wherever possible, 30 measurements (x 1000 magnification) of all taxonomically relevant structures were recorded for each species and the extremes are presented in parentheses. Colony colour was described using notations in the Munsell®Soil Color Charts (Gretag Macbeth, New Windsor, New York, revised 2000). Measurements of conidial size were obtained using the image analysis software Olysia BioReport 3.2 software imaging system. Data analyses were performed using descriptive statistics in Microsoft Excel.

RESULTS

Distribution, impact and symptoms of Kirramyces *sp.*

The disease was first detected in August 2005 causing leaf blight to *E. grandis* and *E. grandis* x *E. camaldulensis* hybrids at a single site in Mareeba, north Queensland. Infection was limited to the lower canopy and no defoliation was observed. The site at Mareeba was revisited in August 2006 and defoliation levels of 95% and greater were noted on the *E. grandis* x *E. camaldulensis* hybrids (Figure 1). New shoots were infected with both *K. epicoccoides* and the *Kirramyces* sp. considered in this study. *Kirramyces epicoccoides* was also detected on the leaves of *E. grandis* and *E. pellita*. While the hybrids were heavily infected with the *Kirramyces* sp., the damage on *E. grandis* of the Copperload provenance was limited to the lower canopy with less then 15% of foliage affected. Several other taxa trials in the region, which included *E. grandis* and *E. grandis* x *E. camaldulensis* hybrids, were surveyed but the *Kirramyces* sp. found at Mareeba, was not detected.

Symptoms on leaves of *E. grandis* and *E. grandis* x *E. camaldulensis* were very similar to those caused by *K. destructans* (Figure 2a, 2d). Necrotic spots appeared on both sides of the leaves and were circular to irregular, 3-20 mm in diam., single to confluent, medium brown to light brown with red brown borders on the adaxial surface, and a light brown colour on the abaxial surface. In some cases, young leaves were severely distorted. Conidia accumulated on the lower surface of the lesions giving rise to crusty black masses (Figure 2c, 2f).

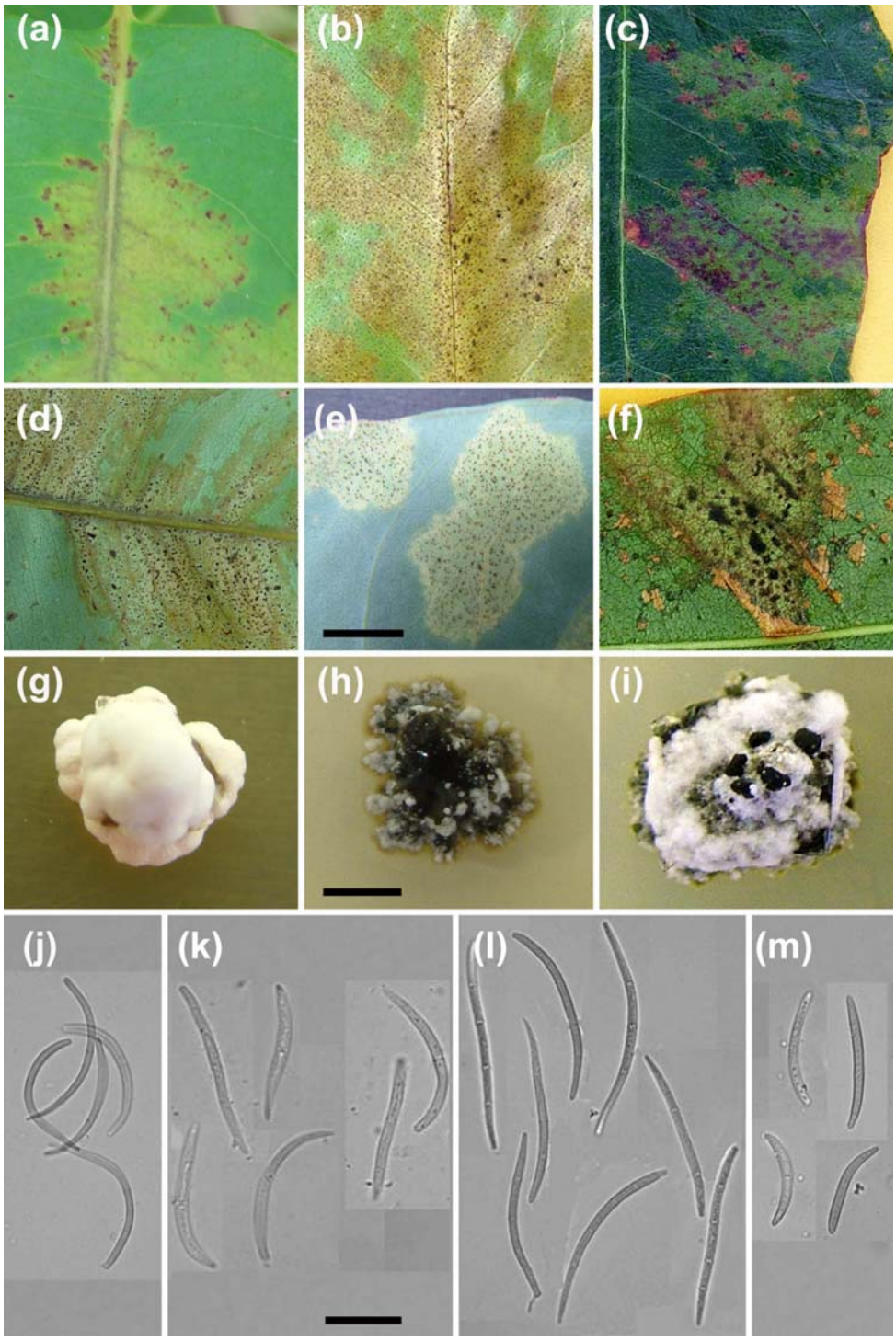


Figure 2. A comparison between the foliar disease symptoms and cultural morphology of three *Kirramyces* spp. Symptoms of (a,d) *K. destructans* on leaves of *Eucalyptus urophylla* hybrids from Guangdong Province, China (b,e) *K. eucalypti* on leaves of *E. grandis,* from Queensland, Australia and (c,f) *K*. viscidus sp. nov on leaves of *E. grandis* from Mareeba, Queensland, Australia. Upper photo, adaxial surface; lower photo, abaxial surface. Bar = 10 mm. Cultures on MEA of (g) *K*. *destructans* isolate CMW 17918 (h) *K. eucalypti* isolate MUCC 384 and (i) *K. viscidus* isolate CBS 121156 Bar = 5 mm. Conidia of (j) *K. destructans* specimen PREM 59261, (k) *K. eucalypti* specimen MURU 425 and (l) *K. viscidus* specimen BRIP 49804 and (m) *K. viscidus* isolate CBS 121156. Bar = 15 µm.

DNA sequence comparisons

The ITS2 data set consisted of 203 characters, of which 51 were parsimony- informative and were used in the analysis. These data contained significant phylogenetic signal (P< 0.01; gl= - 0.88). Heuristic searches of unweighted characters in PAUP resulted in a single most parsimonious tree of 106 steps consistency index (CI=0.65), retention index (RI=0.88). Bayesian analysis resulted in a tree with the same topology and clades as those revealed in the parsimony tree (Figure 3A, TreeBASE=SN3318). The EF 1-α data set consisted of 337 characters, of which 156 were parsimony informative and were used in the analysis. These data contained significant phylogenetic signal (P< 0.01; gl= - 0.93). Heuristic searches of unweighted characters in PAUP resulted in a single most parsimonious trees of 106 steps (CI=0.72, RI=0.89). Bayesian analysis resulted in a tree with the same topology and clades as the parsimony tree (Figure 3B, TreeBASE=SN3318). The β-tubulin data set consisted of 384 characters, of which 90 were parsimony informative and were used in the analysis. These data contained significant phylogenetic signal (P< 0.01; gl= - 0.80). Heuristic searches of unweighted characters in PAUP resulted in a single most parsimonious tree of 106 steps (CI=0.72, RI=0.88). Bayesian analysis resulted in a tree with the same topology and clades as the parsimony tree (Figure 3C, TreeBASE=SN3318).

In all three analyses, the *Kirramyces* sp. from far north Queensland was closely related to, but phylogenetically distinct from *K. destructans* (Figure 3). Isolates of both *K. destructans* and the *Kirramyces* sp. were monomorphic with no sequence variation observed for all isolates tested. Across the three gene regions sequenced, there were 12 fixed polymorphisms distinguishing *K. destructans* from the *Kirramyces* sp. (Table 2).

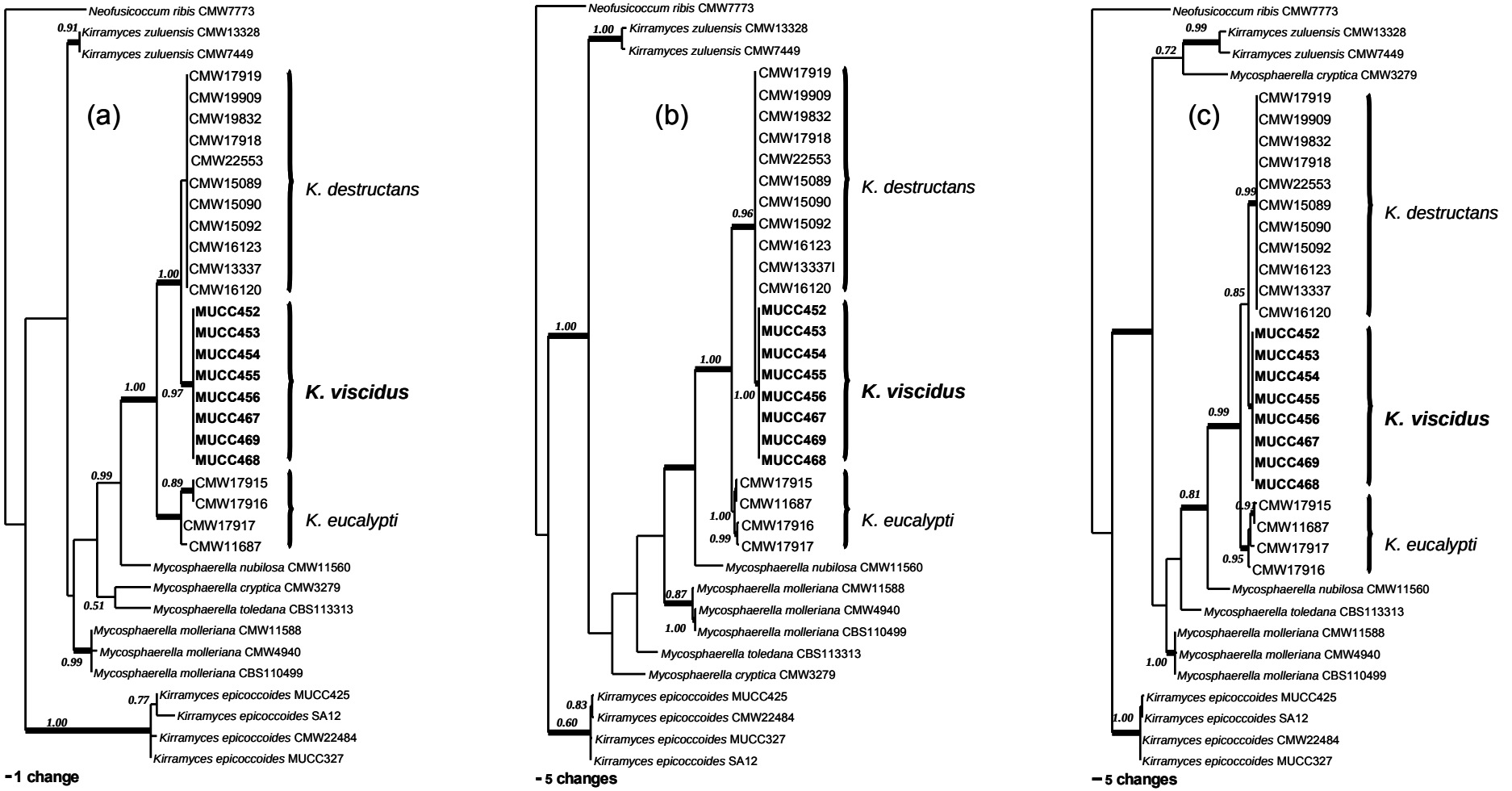


Figure 3. A phylogram of (A) the most- parsimonious tree of 106 steps obtained from the ITS2 sequence data (B) the most parsimonious tree of 439 steps obtained for elongation 1-α sequence data and (C) one of 5 most parsimonious trees of 237 steps obtained from the β-tubulin sequence data. Branches with greater than 75% bootstrap support are thickened. Posterior probabilities of the branch nodes based on Bayesian analysis are given above the branch. All trees are rooted to *Neofusicoccum ribis*. *Kirramyces viscidus* resides in a strongly supported terminal clade close to *K. destructans* in all three gene trees.

Table 2. Polymorphic nucleotides from sequence data of ITS, EF-1α and ß-tubulin gene regions showing the variation between isolates of *Kirramyces destructans* and *Kirramyces* sp. nov.

	ITS			EF-1α				β-tubulin				
	155	194	226	211	228	295	296	131	134	222	224	270
					Kirramyces destructans							
CMW17919	G	C	C	C	C	-	-	A	G	C	G	C
CMW19909	G	C	C	C	C	-	-	A	G	C	G	C
CMW17918	G	C	C	C	C	-	-	A	G	C	G	C
CMW22553	G	C	C	C	C	-	-	A	G	C	G	C
CMW19832	G	C	C	C	C	-	-	A	G	C	G	C
CMW15089	G	C	C	C	C	-	-	A	G	C	G	C
CMW15090	G	C	C	C	C	-	-	A	G	C	G	C
CMW15092	G	C	C	C	C	-	-	A	G	C	G	C
CMW16123	G	C	C	C	C	-	-	A	G	C	G	C
CMW13337	G	C	C	C	C	-	-	A	G	C	G	C
CMW16210	G	C	C	C	C	-	-	A	G	C	G	C
					Kirramyces viscidus							
MUCC452	T	T	T	T	A	T	G	G	A	T	A	T
MUCC453	T	T	T	T	A	T	G	G	A	T	A	T
MUCC454	T	T	T	T	A	T	G	G	A	T	A	T
MUCC455	T	T	T	T	A	T	G	G	A	T	A	T
MUCC456	T	T	T	T	A	T	G	G	A	T	A	T
MUCC468	T	T	T	T	A	T	G	G	A	T	A	T
MUCC469	T	T	T	T	A	T	G	G	A	T	A	T
MUCC470	T	T	T	T	A	T	G	G	A	T	A	T

Morphological characterisation

At first appearance, the conidia of the *Kirramyces* sp. were very similar to those of *K. destructans*. The first difference noted between the *Kirramyces* sp. and *K. destructans* was the extremely hydrophobic and sticky spores and these required 2-4 hr of re-hydration before the conidial masses could be teased apart. This is in contrast to *K. destructans* and *K. eucalypti*, where conidia are easily separated after re-hydration for 1-5 min. The conidia of the *Kirramyces* sp. were very similar to those of *K. destructans* and *K. eucalypti* in pigmentation, shape, size and number of septa (Figure 2j, 2k, 2l, 2m).

Conidia of the new *Kirramyces* sp. were sub-hyaline to pale brown, 0–3-septate and longer (54 µm on average, range 47-60 µm) than those of *K. eucalypti* (mean 43 µm, range 35-50 µm, which are also 0-3 septate) (Heather 1961; Walker *et al*. 1992, Chapter 2). The conidia of the *Kirramyces* sp. were also longer than the conidia of specimens of *K. destructans* collected from China and Indonesia (mean 47

µm, range 38-55µm, 1-3 septate) that were found in Chapter 3 but slightly shorter (50-65 µm. 1-3 septate) than conidia of specimen of *K. destructans* collected from Indonesia that were given by Wingfield *et al*. (1996).

Pycnidia on leaf material collected at Mareeba were over-mature and it was not possible to obtain measurements of conidiogenous cells from these specimens. New collections at the same location were made, but all trees were more then 95% defoliated and the remaining leaves were covered with *K. epicoccoides* mixed with over-mature pycnidia of the *Kirramyces* sp. Thus, the only description and measurements obtained from leaf specimens were those for the conidia. Consequently, pycnidia produced in culture were used to characterise the morphology of conidiogenous cells.

After 30 days of growth at 28° C in the dark on MEA, colonies of the *Kirramyces* sp. were 20-31 mm diam., the upper surface was white to pinkish white with sectors of light greenish grey and they were covered with pycnidia, the lower surface was pinkish grey to reddish brown with irregular margins (Figure 2i). These characteristics are typical of both *K. destructans* and *K. eucalypti* (Crous 1998) (Figure 2g, 2h). On OMA, colonies were 20-29 mm diam., pinkish white on the upper surface, and pink to dark olive grey with a regular margin on the reverse side. On TWA, colonies grew 18-28 mm diam., white to pink on the upper surface with sectors of reddish brown, and olive grey below with a regular margin. Some colonies produced pycnidia on their surfaces. Growth was slower at 20ºC on all three media, though the colour and shape on each was similar. Conidia from culture were shorter (33-45 µm) than conidia measured from leaf material (47-60 µm). This is also true for *K. eucalypti* where conidia produced in culture were shorter (24-32 µm) than conidia produced on leaf material (35-50 µm) (Chapter 3). Of the five isolates used in this study, only one produced pycnidia and conidiogenous cells in culture on MEA at 28ºC.

Taxonomy

Phylogenetic inference and to a lesser extent morphological characteristics, have provided robust evidence that the *Kirramyces* sp. causing a serious leaf disease on hybrids of *E. grandis* x *E. camaldulensis* at Mareeba represents a unique taxon. The fungus is described here as a new species:

Kirramyces viscidus Andjic, P.A. Barber, T.I. Burgess sp. nov. Figure 4

Mycobank no. MB 510859

Teleomorph: ***Mycosphaerella*** sp. (based on phylogenetic inferences, but not seen)

Etymology: Name refers to the hydrophobic and exceptionally sticky conidia of the fungus.

Conidiomata pycnidialia hypophylla, solitaria, atrobrunnea ad atra. *Conidia* solitaria, 0–3-septata, subhyalina et pallide brunnea, parum verruculosa, cylindracea, recta ad diverse curvata, parietibus crassis, ad basim truncata, margine interdum fimbriato, apice obtuso, (30.5-)47-60(-78.5) x (2-)2.5-3.5(4.0-) μm.

Leaf spots: circular to irregular, 3-20 mm diam., single to confluent, medium brown to light brown with red brown border on the top surface, light brown at the bottom. *Conidiomata*: pycnidial, hypophyllous, single, dark brown to black. Conidiophores reduced to conidiogenous cells. *Conidia*: solitary, 0-3 septate, sub-hyaline to pale brown, slightly verruculose, cylindrical, straight to variously curved, thick-walled, base truncate sometimes with marginal frill, apex obtuse, (30.5-)47-60(-78.5) x (2-)2.5-3.5(4.0-) (mean= 54 x 3μm).

Cultures: Colonies 29 x 29 mm after 1 month at 28° C in the dark on MEA, white yellow red 5YR 8/1 to pink 5YR 8/4 on the upper surface, olive grey 5YR 7/1 on reverse. *Mycelium*: Sub-hyaline to pale brown, septate, branched. *Conidiomata* pycnidial, single, dark brown to black, globose to sub-globose, unilocular: wall of *textura angularis*. *Conidiogenous cells*: doliiform to sub-cylindrical, smooth to slightly verruculose, aseptate to 1-septate, (5.5-)8-10.5(-12.5) x (3-)4.5-8(-9.0) (mean= 6.4 x 9.5) μm, sub-hyaline to pale brown, proliferating enteroblastically, 1-2 times percurrently. *Conidia*: solitary, 0-1-septate, sub-hyaline to pale brown, smooth to slightly verruculose, cylindrical, straight to variously curved (29-)35-40(-47.5) x (2.0-)2.5-3.5(-4.0) (mean=37 x 3.0 μm), lateral branches present as secondary conidia, mycelium in culture produce a synanamorph resembling chlamydospores (12.5 x 11μm) that are dark brown and thick walled.

Holotype: on leaves of *E. grandis* Hill ex Maiden, Mareeba, Queensland, Australia, T.I. Burgess, G. E. StJ Hardy, A.J. Carnegie, G. Pegg, August 2005, (HOLOTYPE BRIP 49804; culture ex-type CBS 121157).

Hosts: E. grandis, *E. grandis* x *E. camaldulensis*

Geographic distribution: Mareeba, north Queensland.

Additional specimens examined: K. viscidus on *E. grandis*, Mareeba, Queensland, Australia, T.I. Burgess, G. E. StJ Hardy, A.J. Carnegie, G. Pegg, August 2005, (MURU427; culture ex-isotypes, CBS 121155, CBS 121156, MUCC 454, MUCC 455) and *E. grandis* x *E. camaldulensis*, (MURU431; culture ex-paratypes, MUCC 467, MUCC 468, MUCC 469).

Notes:Kirramyces viscidus can be distinguished from *K. destructans* and *K. eucalypti* by the production of highly hydrophobic and viscous spore masses. *In vivo*, *K. viscidus* produces longer conidia (47-60 μm) than those of *K. eucalypti* (35-50 μm). In contrast to *K. destructans,* whose conidia are 1-3-septate, the conidia of *K. viscidus* are 0-3 septate. Unlike *K. destructans, K. viscidus* produces a synanamorph with chlamydospore-like structures in culture. The conidia of *K. viscidus* produced *in vitro* were shorter 35-40, (mean=37 x 2.8 μm) than those produced *in vivo* 47-60, (mean= 54 x 3μm) (Figure 2m).

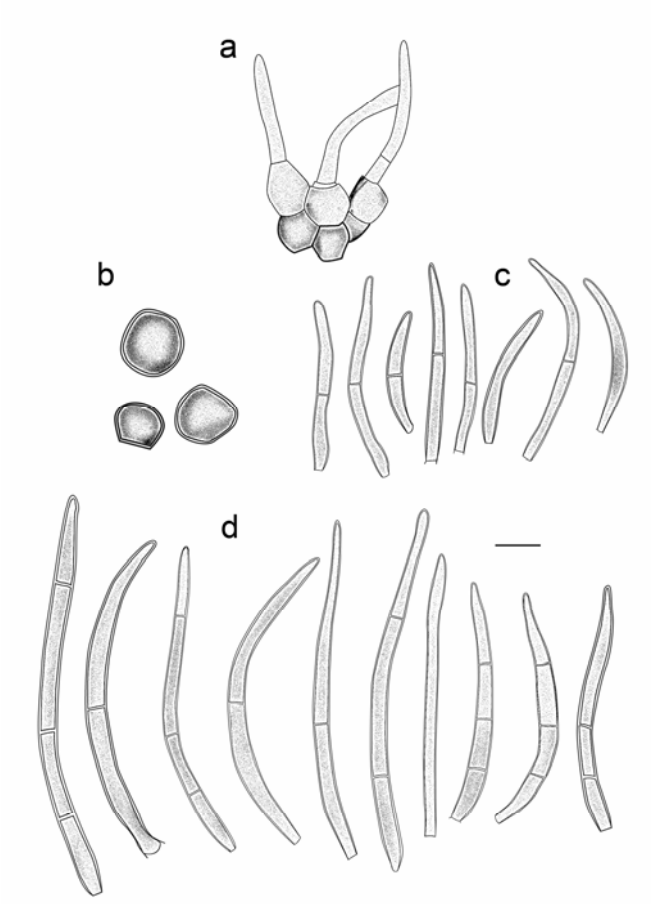


Figure 4. *Kirramyces viscidus* (a), conidiogenous cells and (c) conidia produced on MEA, (b) mycelium in culture producing chlamydospore- like synanamorph, (d) conidia produced *in vivo*. Scale bar = 10 μm.

DISCUSSION

Comparisons of DNA sequence data revealed that the *Kirramyces* sp. collected from leaves of *E. grandis* and the hybrid of *E. grandis* x *E. camaldulensis* from Mareeba, far north Queensland, represents a new taxon. This fungus has been named *K. viscidus*, a name emphasising the stickiness of its conidia. Based on phylogenetic analyses of sequence data obtained for the ITS, EF-1α and β-tubulin gene regions, *K. viscidus* has 12 polymorphic sites, which distinguish it from the closely related *K. destructans.* Based on morphological characteristics, *K. viscidus* can be distinguished from *K. destructans* by its remarkably sticky and hydrophobic conidia and the number of septa in the conidia. This report represents the first record of a new *Kirramyces* species very closely related to the destructive leaf blight and shoot pathogen, *K. destructans.* The latter fungus is considered a serious threat to the biosecurity of native eucalypts (Burgess *et al.* 2006a) but has not been detected in Australia. The presence of a very similar but different fungus in the country will necessitate very careful comparisons when new records of similar *Kirramyces* spp. are made.

Sequence comparisons for all the isolates of *K. viscidus* examined in this study were identical. Conidial measurements for specimens on leaves also revealed no obvious differences between different collections. *Kirramyces* spp. from eucalypts are morphologically similar and thus difficult to distinguish from each other. Their current taxonomy consequently relies more heavily on DNA sequence comparisons than on morphology (Chapters 2 and 3).

The eucalypt taxa trial where *K. viscidus* was discovered was established in an ex-pasture area near remnant vegetation. In this trial, the *E. grandis* x *camaldulensis* hybrids from South America are highly susceptible to infection by *K. viscidus,* while endemic *Eucalyptus* spp. are less susceptible. These hybrids, a range of clones initially tested in the late-1990s, have also shown high susceptibility to *K. epicoccoides* in New South Wales (Carnegie 2007b) and to *K. eucalypti* in southern Queensland (Pegg, pers. comm.). They would, therefore, not be suitable for planting in far north Queensland. This is not surprising, as these hybrids were specifically selected for planting in South America; an area free of most Australian native pests and diseases to which they may be susceptible. *Eucalyptus grandis* selected from seed derived from the Copperload provenance near Cairns, in north Queensland

was less susceptible to infection by *K. viscidus*, with only 15% of the foliage affected. *K. viscidus* was not detected on *E. pellita* and *Corymbia* spp. also planted in the trial. Based on these findings, *K. viscidus* is most probably endemic to Australia and would be unlikely to cause serious diseases on endemic *Eucalyptus* spp. The most likely source of the pathogen is the remnant vegetation close to the trial site.

The close relatedness of *K. viscidus* to the aggressive pathogen *K. destructans* and the similarity in disease symptoms, suggests that the pathogen could seriously damage Australian eucalypt plantations in the future. There is an urgent need to study the biology and ecology of *K. viscidus* and to determine its potential impact to the eucalypt industry. Thus, surveys to find possible hosts in both native forests and plantations need to be conducted in tropical regions, where non-endemic eucalypt plantations are being established. Here, priority should be given to native stands of *E. grandis* to target potential resistance for breeding programs using Australian derived material.

Chapter 7

General Discussion

Phaeophleospora destructans is a devastating pathogen described from Sumatra in 1996 (Wingfield *et al*. 1996). At the commencement of this project it was known from Sumatra, East Timor, Vietnam and Thailand, but not present in Australia and was the subject of a research project at Murdoch University examining new and emerging pathogens threatening the biodiversity of Australian eucalypts. During the present study several populations of *P. destructans* were collected in Asia. The examination of isolates from China revealed for the first time that *P. destructans* is present in China (Appendix I).

Initial studies to resolve the relationship between *P. destructans* and other *Phaeophleospora* spp. based on DNA sequencing, encountered some problems regarding the phylogenetic placement of this pathogen given in the literature (Crous *et al*. 2001). DNA sequences obtained in the present study showed that *P. destructans* was distant (87% sequence homology) from the *P. destructans* sequence obtained from GenBank but was closely related to other *Phaeophleospora* spp. from eucalypts.

This finding led to further examination and comparison of *Phaeophleospora* spp. with the type specimens and included two new species found on eucalypts that resembled *Phaeophleospora* spp. and *Colletogloeopsis* spp. Some unexpected problems were found when trying to accommodate these latter two species, as one of them could not be accommodated in either *Phaeophleospora* or *Colletogloeopsis* as it produced conidia that by morphological description fell between these two genera. However, phylogenetic analysis placed these two new species and all other species of *Colletogloeopsis* and *Phaeophleospora* isolated from eucalypts in the same strongly supported clade. This clade included *Mycosphaerella* spp. with *Colletogloeopsis* anamorphs such as *M. cryptica*. As a result, the genus *Kirramyces* (which has priority over *Colletogloeopsis*) was resurrected and all *Phaeophleospora* and *Colletogloeopsis* spp. from eucalypts were transferred to *Kirramyces*, including *P. destructans*. The interesting thing about this phylogenetic clade is that the four most devastating *Mycosphaerella* pathogens of eucalypts, *M. cryptica*, *M. nubilosa*, *K. destructans* and *K. eucalypti* all reside in the same clade.

Once the taxonomic position of *K. destructans* was resolved, phylogeographical studies of Asian populations were performed to track the origin of the pathogen and to follow the pathway of its movement throughout Asia. This study showed that isolates from Asia have low genetic diversity,

which strongly suggests that *K. destructans* is not endemic to those parts of Asia where the collections were made, and was introduced from elsewhere, most likely on infested germplasm.

In association with the research project at Murdoch University that included surveys in Cairns, FNQ, *K. eucalypti* and a fungus resembling *K. destructans* were found. This is the first report of *K. eucalypti* in northern Australia. Examination of the species resembling *K. destructans* led to the description of a new species named *K. viscidus*. *K. viscidus* is morphologically and phylogenetically similar to *K. destructans* thus, is of concern for the management of eucalypt clonal and off-site plantations in Australia.

Because of the distribution of *K. eucalypti* and the recent impact it was having on plantation species in central QLD and southern NSW, and its close relationship to *K. destructans* and *K. viscidus*, it was decided to examine this species in more detail. The aim of this part of the study was to determine the origin and movement of *K. eucalypti* in Australia and New Zealand. Two major issues emerged from this study. Firstly, isolates from Queensland were phylogenetically different (with nine polymorphic sites across three gene regions) to isolates from elsewhere, thus they may represent a new species or even a hybrid. Secondly, the highest genetic diversity was found in isolates from New South Wales, not those from Victoria where the pathogen was first reported.

Just at the completion of this PhD project, *K. destructans* was isolated from *E. grandis* x *E. urophylla* clones planted in a taxa trial on Melville Island, northern Australia. In addition, an isolate from Derby, Western Australia was identified as *K. destructans* using molecular and morphological characteristics, although the isolate was from adult leaves and the symptoms did not resemble those generally caused by *K. destructans*. These isolates comprise the first report of *K. destructans* in Australia (Appendix II). Population studies of *K. destructans* from Australia are underway. Initial results have shown the diversity of the *K. destructans* population from Australia to be much higher than the populations from Asia. The data suggest that *K. destructans* is endemic to Australia in native vegetation, where it does not cause noticeable disease, and only expresses itself when non-endemic eucalypts are planted. Traditionally, northern Australia has not been seen as an area suitable for eucalypt plantations and thus there have been very few trials of plantation eucalypts in the region. However, as plantations are

established in the region, it is likely that many diseases will be found, especially on trees that are hybrids or for trees planted off site.

Kirramyces in Australia

Over the past four years, surveys for pests and diseases were conducted on eucalypts in northern Australia (Burgesss, Carnegie, Pegg, Whyte, pers. comm.). These plantations have been recently established and act as sentinel plantings as the eucalypt species in the trials are not endemic to the region. During these surveys many undescribed *Kirramyces* spp. have been found (Burgess, Carnegie, Whyte, pers. comm.). This suggests that *Kirramyces* spp. are common in northern Australia. Many of these new species have short conidia while a few have long conidia, but phylogeneticaly they all reside in a well-resolved monophyletic clade (Appendix III, Figure 1).

Crous *et al.* (2007) have recently described a new species from *Eucalyptus* named *Phaeophleospora stonei* that is morphologically similar to *Kirramyces* spp. with long conidia. On GenBank, *P. stonei* was closest to the newly described *M. pseudovespa* (Carnegie *et al*. 2007) and *M. walkeri* (Crous *et al*. 1995). In phylogenetic analysis, *P. stonei* was basal to the "nubilosa" clade with 100% bootstrap support as an individual species (Appendix III). As this single species emerged in a separate clade to all others, Crous *et al.* (2007) concluded that *Phaeophleospora* is polyphyletic. This is in contrast to findings of the present work as all *Kirramyces* spp. (formerly *Phaeophleospora*) reside in the same strongly supported monophyletic clade. The contrast between the two studies could be due to the difficulty of obtaining pure cultures of isolates from a single lesion. For example, from leaf material obtained in tropical or sub-tropical Australia it is very difficult to isolate *Kirramyces* spp. This is because the high humidity is conducive to fungal growth and the specimens are often contaminated with many other fungi. In addition, multiple *Mycosphaerella* species are found to occupy the same lesion (Andjic, Barber, Burgess, Carnegie, Whyte, unpublished data) and often the fungi identified from lesions by molecular studies did not appear to correspond with initial morphological identification. Therefore, it is possible that 'matches' between initial morphological identification and subsequent DNA analyses are not the same. Thus, an examination of the herbarium specimen and culture of *P. stonei* needs to be made before the theory of a monophyletic clade for *Kirramyces* is

abandoned. Attempts have been made to obtain this culture and herbarium specimen, but although published, they are not yet available in a public data base.

Future research

Based on the findings from this study, future research should focus on closer examination of *Kirramyces* spp. as they are already causing serious damage in eucalypt plantations in the sub-tropics and tropics of Australia. In addition, some of the new species are closely related to the serious stem canker pathogens, *K. zuluensis* (Cortinas *et al*. 2006b) and *K. gauchensis* (Cortinas *et al*. 2006c). Those pathogens have not been found in the areas of origin of *Eucalyptus* (Australia and northern neighbors). Like *K. destructans*, *K. zuluensis* may be native to Australia, but has not as yet been found. They also belong to the same phylogenetic clade as the most devastating *Mycosphaerella* pathogens of eucalypts and are thus, of high concern for the establishment of eucalypt plantations.

The origin of the devastating pathogen, *K. destructans* still needs to be determined. This could not be answered in the present study as isolates from Asia showed low genetic diversity suggesting that *K. destructans* was introduced to Asia. Recently, *K. destructans* has been found on *E. urophylla* x *E. grandis* a non-native eucalypt on Melville Island, northern Australia and on a *Eucalyptus* sp. in Derby, western, Australia which confirmed our hypothesis of its presence in Australia. Future surveys should focus on native vegetation on Melville Island and adjacent to eucalypt taxa trials in north Queensland to determine if *K. destructans* is present in native vegetation in tropical Australia. Besides Timor, where *K. destructans* has been reported, there are native eucalypts, *E. deglupta*, *E. alba* and *E. urophylla*, in east Indonesia and Papua New Guinea. The susceptibility of *E. urophylla* strongly suggests that this species has not co-evolved with *K. destructans*. However, *K. destructans* may still be found on other endemic eucalypts in the region. Thus, if possible, isolates of *K. destructans* from East Timor should be obtained and endemic eucalypts in Papua New Guinea should be surveyed. The origin of *K. destructans* should then be revealed through multi gene genealogies including microsatellite markers and amplified fragment length polymorphism (AFLP).

Very little is known about the biology and epidemiology of *Kirramyces* spp. A study monitoring the seasonal development of the disease in response to *K. eucalypti* infection at sites with different

environmental conditions was conducted in New Zealand after the outbreak of disease caused by this pathogen (Hood *et al*. 2002a, 2002b). The results obtained by Hood and others have shown that conidia of *K. eucalypti* are present on leaves throughout the year but the availability of inoculum was lower during the winter than in spring and summer due to cold conditions. Spore dispersal of *K. eucalypti* is believed to be wind and rainfall dependant. No spores were found in litter although the authors suggested that this warrants further investigation. The findings obtained by Hood *et al*. (2002a, 2002b) are in agreement with the observations of the present study. The major outbreaks of disease caused by *Kirramyces* spp. in Australia have been in periods of high humidity and warm temperature, conditions believed to be favorable for conidial dispersal. Future research should try to determine factors influencing the epidemiology of *Kirramyces* spp. including pathogen survival across seasons, incubation time of spores and host susceptibility. In some *Mycosphaerella* spp. the primary inoculum for epidemics are ascospores (Inmana *et al*. 1999; Hunter *et al*. 1999) but the primary source of inoculum of *Kirramyces* spp. is presently unknown. The presence of *Kirramyces* spp. on seeds has never been examined, but the seed could well be the major source of pathogen survival and responsible for its dispersal between countries via commercial trade (Wingfield *et al*. 2001).

Eucalyptus camaldulensis, *E. globulus*, *E grandis*, *E. pellita*, *E urophylla* and *E. tereticornis* are widely grown plantation eucalypt species (Turnbull 2000) and all are susceptible to *Kirramyces* spp. Therefore, future research should conduct screening trials of common eucalypts to determine susceptibility to *Kirramyces* spp., in particular to the devastating pathogen *K. destructans*. Part of this study is now underway (Burgess, pers. comm.) eucalypt species from a wide distribution in tropical Australia are being tested for their susceptibility to *K. destructans* in the dry tropics in central Thailand, the wet tropics in northern Vietnam and southern China, and a temperate climate in China. This trial is planned to be repeated on Melville Island and compared with trials from Asia. The results from these trials should provide information on the susceptibility of a range of tropical eucalypt species that can be used for production eucalypts.

The teleomorphs of *Kirramyces* spp. are not known or are very rare in nature, although this does not mean that they do not exist. Sexual reproduction and recombination from population studies have been

demonstrated in fungi once thought to be asexual; examples include *Candida albicans* (Hull *et al*. 2000), and *Aspergillus flavus* (Geiser *et al*. 1998). The sexual stage of *K. destructans*, *K. eucalypti* and *K. viscidus* have not been observed but based on phylogenetic analyses should be *Mycosphaerella* (Chapters 2 and 3).

Kirramyces destructans, *K. eucalypti* and *K. viscidus* are morphologically almost indistinguishable and based on multi-gene genealogies are very closely related. Based on sequence data for several gene regions, few polymorphic sites were found to separate the three species. The main difference was found to be an indel in the ITS region of *K. eucalypti*, which was absent in *K. destructans* and *K. viscidus*. However, some isolates of *K. destructans* from Melville Island contain this indel (Burgess, unpubl.) suggesting that the process of hybridization and/or speciation may be involved. Furthermore, *Kirramyces* spp., in particular *K. eucalypti* were found to be more aggressive in NSW and QLD than they were a few years ago (Carnegie, Pegg, pers. comm.). In general, the driving forces of emerging diseases are known to be a combination of factors such as, climate change, monoculture, pathogen pollution, anthropogenic movement of germplasm and host-pathogen evolution (Anderson *et al*. 2004). The cause of a change in pathogenicity of *K. eucalypti* is unknown and deserves further investigation.

Some closely related fungal species have the ability to hybridize (Brasier *et al*. 1995; Brasier *et al*. 1998; Tsai *et al*. 1994; O'Donnell & Cigelnik 1997). Hybrids of plant pathogenic fungi can be devastating as they often have a new or greater host range then either parent (Brasier *et al*. 1999; Newcombe *et al*. 2000) and could have huge ecological and economic importance such as *Phythophtora alni* subs. *alni* (Brasier *et al.* 2004). *Phythophtora alni* is the hybrid of *P. cambivora* and a species close to *P. fragariae*, which caused outbreaks of a new alder disease in southern Britain where more than 10,000 alder trees were killed. Since then it has spread to other parts of Europe causing severe damage (Gibbs *et al*. 2003; Jung *et al*. 2003; Jung & Blaschke 2004).

Another intriguing example of hybridization is Duch elm disease, responsible for destruction of elm trees on a global scale. The epidemic of this disease occurred twice in two major waves; the first epidemic was caused by *Ophiostoma ulmi* in 1900s and the second was caused by *O. novo-ulmi*

(Brasier *et al*. 1998). During the examination of a large collection of isolates (11.000) from Eurasia and North America, isolates resembling both *O. ulmi* and *O. novo ulmi* were found. Molecular analysis reveled that those isolates were hybrids (Brasier *et al*. 1998; Schardl & Craven 2003). These hybrids were found to be unfit, rare and transient. The authors interpreted them as a bridge between *O. ulmi* and *O. novo-ulmi* where *O. novo-ulmi* is replacing *O. ulmi.*

Hybridization between existing *Kirramyces* spp. could be devastating for the eucalypt plantation industry in Australia and needs to be explored further. For example, if a tree hybrid or clone had been bred to be resistant to existing *Kirramyces* species, it may still be susceptible to a hybrid fungal species. Thus, future research should focus on determining the reproductive strategy, source of variation and potential hybridization between closely related *Kirramyces* spp. To achieve this, a good sampling approach would be crucial, as more new *Kirramyces* spp. were found on recently established plantations in sub-tropical and tropical Australia (Appendix III). Thus, extensive surveys and sampling should be conducted in both native and plantation species in northern Australia. Once a range of isolates are obtained, population studies would be possible. This can be achieved by using gene phylogeny inferred from DNA sequences of different gene regions, microsatellite loci and AFLPs. Microsatellites consist of a specific sequence of DNA bases which contain 1-6 tandem repeats. Microsatellite alleles differ in the number of repeats. In phylogenetic inference, they have been useful in examining geographic division of genetic variation in populations (Fisher *et al*. 2000). AFLP is a PCR based method that is widely used for rapid screening of genetic variation in strains or closely related species. It has been successfully used in population studies of *Phythophthora ramorum* (Ivors *et al.* 2004).

To estimate ancestral relationships among *Kirramyces* spp. a combination of maximum parsimony, Bayesian and coalescent methods should be applied. The maximum parsimony method is used to reconstruct phylogenetic trees from sequences. It works on the principle of finding the shortest tree, *i.e.* the tree with the smallest number of changes that explains the observed data. Bayesian methods address phylogenetic uncertainty by averaging inferences of evolutionary processes and parameter estimates over all possible phylogenies (Huelsenbeck *et al*. 2000). The coalescent method is a

retrospective model of population genetics that traces all alleles of a gene in a sample from a population to a single ancestral copy shared by all members of the population. The data should then reveal evolutionary mechanisms (recombination, mutation, parasexuality, hybridization, origin, movement) behind the observed variation in closely related *Kirramyces* spp. This knowledge in combination with plant stock genetics can be used to predict durability of resistance that may be expected in planting stock.

Conclusions

- The previous genus name *Kirramyces* has been resurrected for *Phaeophleospora* spp. which specifically occur on eucalypts.
- *Phaeophleospora* and *Colletogloeopsis* spp. from eucalypts have been transferred to the newly resurrected genus *Kirramyces*.
- *Kirramyces* spp. occurring on eucalypts are common in Australia and are all closely related and well placed in a strongly supported monophyletic clade. Their incidence and severity is increasing thus studies at the population level are essential.
- The devastating pathogen, *Kirramyces destructans* is most likely native to Australia and has been spread to Asia via human-mediated movement of germplasm.
- New South Wales is a probable source of origin of *K. eucalypti*, but needs to be investigated further.
- Three new *Kirramyces* spp. have been described and many more are waiting to be described.
- The epidemiology of *Kirramyces* spp. needs to be investigated further.
- Prior to planting non endemic eucalypts, a test for their susceptibility to native and exotic pathogens is essential.

APPENDIX I

First report of *Phaeophleospora destructans* in China

Published as: Burgess TI, Andjic V, Hardy GEStJ, Dell B, Xu D, 2006. Firs report of *Phaeophleospora destructans* in China. *Journal of Tropical Forest Science*, **18**: 144-146

Eucalypts are highly favored plantation species as they are fast growing and easy to cultivate. The timber is an important source of fiber to the international paper and pulp industry. Plantation forestry in China is rapidly expanding, and now exceeds more than 1.3 million ha, mostly *Eucalyptus urophylla, E. grandis* and their hybrids (Minsheng 2003; Qi 2003). A number of foliar plant pathogens have been reported to impact on yields in plantations of eucalypt species in tropical Asia including *Mycosphaerella* spp., *Phaeophleospora* spp., *Cryptosporiopsis* spp. and *Cylindrocladium* spp. (Barber 2004; Old *et al.* 2003b). In this study, we report for the first time the presence of *Phaeophleospora destructans* in China. Six *Phaeophleospora* species are known to cause disease on eucalypts; *P. delegatensis, P. lilianiae, P. epicoccoides, P. eucalypti* and *P. destructans* and the newly described *P. toledana* (Crous 1998; Crous *et al.* 2004). Of these, *P. delegatensis, P. lilianiae* and *P. toledana* are limited in their distribution, while *P. epicoccoides, P. eucalypti* and *P. destructans* are considered major eucalypt pathogens (Hood *et al.* 2002a; Park *et al.* 2000; Wingfield *et al.* 1996).

Phaeophleospora epicoccoides, P. eucalypti and *P. destructans* all cause leaf blights and discoloration of the lower crowns that leads to premature defoliation, reduced growth and vigour, and in some instances tree death within plantations. *Phaeophleospora epicoccoides* is found wherever eucalypts grow, whilst the distribution of *P. eucalypti* is restricted to Australia and New Zealand (Park *et al.* 2000). *Phaeophleospora destructans,* the most aggressive species, was first described from 1 to 3-year-old *E. grandis* in Sumatra, Indonesia (Wingfield *et al.* 1996). Since then it has been found in Thailand, central and northern Vietnam and East Timor (Old *et al.* 2003a; Old *et al.* 2003b). Besides *E. grandis, P. destructans* has been isolated from other eucalypts including clones of *E. camaldulensis* and *E. urophylla* (native in East Timor). *Phaeophleospora destructans* has not been found in Australia and its potential impact on native eucalypt forests is unknown.

Between November 2003 and July 2004, production nurseries were inspected for the incidence of nursery foliar pathogens in four eucalypt growing regions of China; Longtan, Guang Xi Province, Kaiping and Leizhou Peninsula, Guangdong Province, Simai, Yunnan Province and Haikou, Hainan Province (one nursery in each Province). Leaf blight was observed in all nurseries in all regions. Leaf blight was also observed in young plantations in Guandong and Guang Xi Provinces. Many leaves had

dropped, but early symptoms could be seen (Figure 1a-d). These included large chlorotic lesions not delineated by veins with masses of black fruiting bodies on the undersurface of the leaves. These symptoms are typical of *Phaeophleospora destructans* although microscopic examination of the spores revealed *P. epicoccoides* fruiting in some of these lesions. This was unexpected as *P. epicoccoides* is generally infects older leaves. We have since seen these symptoms (caused by *P. epicoccoides*) on seedlings in South Africa and Colombia (unpublished data). In some young plantations on the Leizhou Peninsula, vein-limited purplish lesions were observed on older leaves, these are typical of *P. epicoccoides* (Figure 1). In some cases, symptoms indicative of both pathogens were observed on the same tree.

Phaeophleospora spp. were isolated in the following manner. Under the dissecting microscope, spores oozing from single pycnidia were collected on the tip of a sterile needle. The spores were placed on malt extract (20 g l^{-1}) agar (MEA), in a single spot and allowed to hydrate for 5 min. Under the dissecting microscope, spores were then streaked using a sterile needle and single spores picked off the agar and transferred to new MEA plates. Spores usually germinated within 24 hours. Cultures were maintained at 25 ºC.

Cultures of *P. destructans* and *P. epicoccoides* differ in colour and texture. Those of *P. epicoccoides* are dense, slow growing and dark while those of *P. destructans* are pinkish, slow growing and slightly fluffy (Figure 1e-f). In comparison to other species, *Phaeophleospora epicoccoides* has short, wide, multiseptate spores, they are olive green in color whilst spores of *P. destructans* are longer and thinner (Figure 1g-h, Table 1). Spore dimensions of Chinese isolates are within the range of the described species (Wingfield *et al.* 1996; Walker *et al.* 1992).

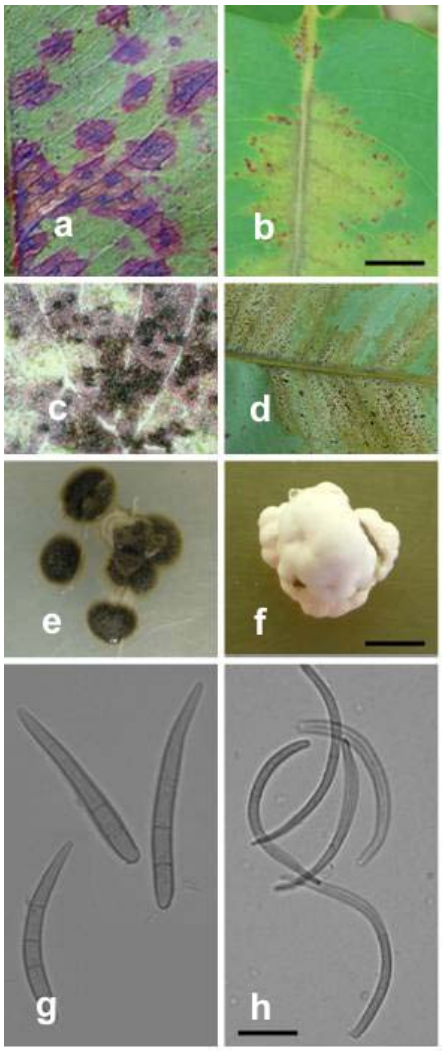


Figure 1. Comparison of the two *Phaeophleospora* spp. found in China Symptoms of *P. epicoccoides* (a) adaxial, (c) abaxial and *P. destructans* (b) adaxial, (d) abaxial on leaves of *E. urophylla* hybrids from Guangdong Province, China. Sporulation on abaxial surface. Bar = 10 mm. Cultures on MEA of (e) *P. epicoccoides* isolate CMW14696, and (f) *P. destructans* isolate CMW17919. Bar = 5 mm. Spores of (g) *P. epicoccoides* isolate CMW14696, and (h) *P. destructans* isolate CMW17919. Bar = 10 µm.

Symptoms, culture characteristics and spore morphology confirmed the presence of *P. destructans* and *P. epicoccoides* in all regions of China visited. *Phaeophleospora epicoccoides* is found predominantly on ageing leaves and, while it may contribute to early leaf drop, it is not considered generally a major pathogen. *Phaeophleospora destructans* is a major pathogen of juvenile foliage in the nursery, on mother plants and in the field. Infected seedlings, mother plants and cuttings in the nursery can be totally defoliated under humid conditions. If infected material survives to be out-planted, this will result in poor establishment and growth.

Table 1. Comparison of spore dimensions between the *Phaeophleospora* spp. found in China. Representative isolates collected in Kaiping, Guandong Province.

Isolate*	Species	Conidia (µm)	Length/Width	Septa
CMW14696	*P. epicoccoides*	19-(29-35)-43 x 2.5-4.3 [Ave. 31.9 x 3.2]	9.9	1-4
CMW17919	*P. destructans*	34-(36-44)-50 x 1.4-2.8 [Ave.40.1 x2.4]	17.0	1-2

* CMW = Culture collection of the Forestry and Agriculture Biotechnology Institute, University of Pretoria, South Africa

Phaeophleospora destructans has only been reported from eucalypts, and as eucalypts are not native (except in east Timor), one possibility is that it is native to Australia and has been introduced elsewhere on germplasm. However, *P. destructans* has not been reported in Australia and currently appears to be restricted to Southeast Asia and China. As the symptoms are obvious and the impact so extensive, it can be assumed that it would have been observed earlier than 1996 (Wingfield *et al.* 1996) if its distribution was extensive. Most likely, it emerged as a major disease for the first time in 1996 in the wet tropics in Northern Sumatra and has been distributed throughout Asia on infected germplasm (seed or cuttings). The present geographically isolated distribution in China suggests that the pathogen has been introduced in recent times and has been dispersed with nursery mother stock. East Timor is a potential origin for the pathogen and its occurrence on native *E. urophylla* and other eucalypts in the region needs to be explored and molecular tools used to identify population structures.

Control of *P. destructans* is difficult. Extensive use of fungicides in the nursery can mask symptoms but will not eradicate the pathogen and the disease will express itself in plantations (Brown 2000). Nursery hygiene and the use of elevated benches with good air flow will also reduce impact in the

nursery. There are some eucalypt plantations in southern China which are free of the disease, but this is not likely to remain for long, given the movement of planting stock and mother plants over long distances in the region. In the long term, the only practical control is to develop resistance within the eucalypt hosts. *Eucalyptus grandis* is particularly susceptible and we recommend the destruction of susceptible clones in favour of hybrids or clones of *E. grandis* showing some tolerance.

This is the first report of *P. destructans* in China. Its identification in most of the established eucalypt growing regions in south China suggests it has either been present for some time or has been recently distributed around the country on infected germplasm. As new commercial nurseries are being established and mother stock is being sourced from older nurseries, it is very important that hygiene measures are implemented to prevent further contamination of planting stock.

APPENDIX II

The eucalypt leaf blight pathogen *Kirramyces destructans* discovered in Australia

Accepted as: Burgess TI, Andjic V, Wingfield MJ, Hardy GEStJ, 2007. The eucalypt leaf blight pathogen *Kirramyces destructans* discovered in Australia *Australasian Plant Disease Notes* **2**: 141-144.

Abstract

Kirramyces destructans is a serious pathogen causing a leaf, bud and shoot blight disease of *Eucalyptus* species in plantations of the subtropics and tropics of South East Asia. This pathogen was first discovered in Indonesia in 1995 and has subsequently spread to Thailand, China and Vietnam. *Kirramyces destructans* is not known to occur in Australia and has been considered a major biosecurity threat. Over the last past four years, surveys have been conducted in existing eucalypt trials in tropical Australia. Several *Kirramyces* spp. were detected in these surveys, including isolates with morphological and cultural characteristics resembling those of *K. destructans.* In this study, DNA sequences of three gene regions were used to compare isolates of *Kirramyces* spp. emerging from the surveys and these were compared with those of *K. destructans* and the closely related *K. eucalypti* and *K. viscidus*. Results have shown, for the first time, that *K. destructans* is present in northern Australia (Melville Island, Northern Territory and Derby, Western Australia). The observed sequence variation among a small number of isolates also strongly suggests *K. destructans* is endemic to Northern Australia.

Kirramyces destructans is an aggressive pathogen first reported causing disease on 1-3 year old *E. grandis* in Sumatra, Indonesia (Wingfield *et al.* 1996). Since then it has been detected in Thailand, China and Vietnam. In these countries it has been found on *E. grandis* as well as *E. camaldulensis* and *E. urophylla* and various hybrids between the three species (Barber 2004; Burgess *et al.* 2006a; Old *et al.* 2003a, 2003b). *Kirramyces destructans* has also been reported from native *E. urophylla* in East Timor (Old *et al.* 2003a).

Figure 1. Leaf blight on 18 month old *Eucalyptus grandis* caused by *Kirramyces destructans* in eastern Guangdong province, China. Leaf blight caused by *K. destructans* has resulted in the loss of all the crown except for the recently emerged leaves which were already infected. Photo B. Dell.

Symptoms of infection by *K. destructans* include distortion of infected leaves and blight of young leaves, buds and shoots. The pathogen causes severe defoliation of juvenile leaves on trees in plantations (Figure 1) and infection of young tissue on clonal mother plants in production nurseries can seriously affect productivity. The pathogen has never been found in Australia, where most *Eucalyptus* spp. are native, but its discovery in East Timor, where *E. urophylla* occurs naturally (Old *et al.* 2003a), suggested this country might represent the area of origin of *K. destructans.* As such, *K. destructans* could have moved into South East Asia on infected germplasm from the substantial

collections of *E. urophylla* from Timor. Due to the devastating impact that *K. destructans* could have on eucalypt plantations and native forests in Australia, this pathogen has been listed on the Plant Biosecurity Watch List for Australia (http://www.daff.gov.au).

Over the last four years, we have been studying the population diversity and distribution of *K. destructans* in Asia and the biosecurity threat this pathogen might pose to eucalypt plantations and forests in Australia. As part of this project, surveys have been conducted in Northern Australia, using existing trials of non-endemic eucalypts as sentinel plantings and by evaluating these trials for disease caused by *Kirramyces* spp. A new species, *Kirramyces viscidus* Andjic, P.A. Barber, T.I. Burgess, which is closely related to *K. destructans*, was discovered in a taxa trial in Northern Queensland (Andjic *et al*. 2007b).

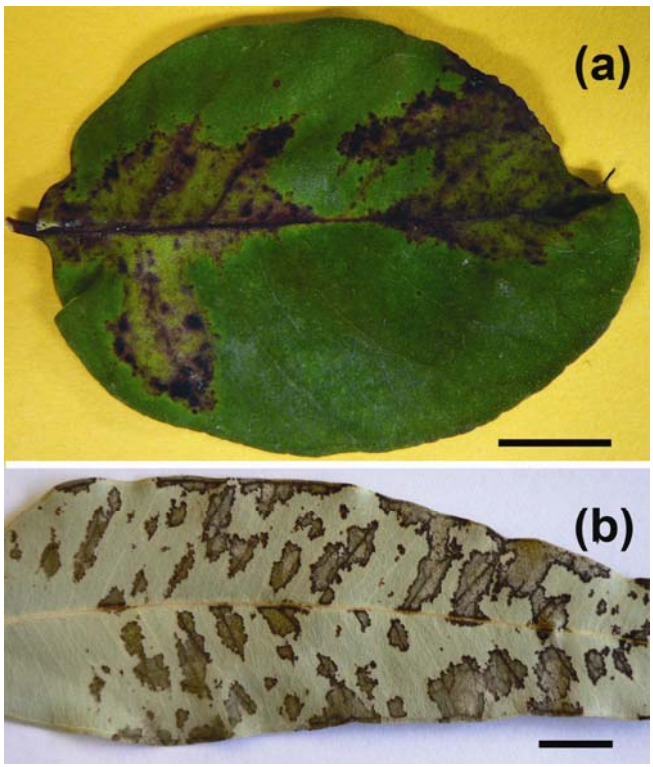


Figure 2. Symptoms of *K. destructans* on (a) juvenile leaves of *Eucalyptus urophylla x E. grandis* on Melville Island, Northern Territory and (b) adult leaves of an unknown *Eucalyptus* species in Derby, Western Australia. Bar = 1cm

Juvenile eucalypt leaves with symptoms resembling those of *K. destructans* (Figure 2a) were collected in July 2006 from a clonal taxa trial on Melville Island, 50 km off the coast from Darwin, Northern Territory, Australia. The trees had been severely damaged by cyclone Ingrid in March 2006 and they were coppiced approximately two months later. The abaxial surfaces of the leaves were covered with

pycnidia, exuding conidia resembling those of various species including *K. destructans, K. eucalypti* and *K. epicoccoides*. Adult leaves were also collected from a mature tree of an unknown *Eucalyptus* sp. at the Kimberly Entrance caravan park in Derby, Western Australia (Figure 2b). Although the symptoms on these leaves were not typical of *K. destructans,* this material was studied because the conidia were similar to those of the pathogen.

Isolations from conidia taken from leaves of trees on Melville Island and those from Derby were made as described previously by Andjic *et al*. (2007a). Cultural characteristics of several isolates were the same as those of *K. destructans,* with white to pink colonies producing black spore masses on the upper surface and olive-green to black at the centres on the reverse sides of the plates. All isolates have been maintained in the Murdoch University culture collection (MUCC) (Table 1).

Genomic DNA was extracted from cultures as described previously by Andjic *et al*. (2007a). Initially, the second internal transcribed spacer and part of 5.8S region of the rDNA (ITS2) was amplified and sequenced for all *Kirramyces* isolates. For those isolates with sequence data similar or identical to *K. destructans,* the β-tubulin (ßt) and translation elongation factor 1α (EF-1α) gene regions were amplified and sequenced as previously described by Andjic *et al*. (2007a). Parsimony analyses were performed on the combined data sets in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2003) and Bayesian analysis was made using MrBayes (Ronquist & Huelsenbeck 2003) following the methods described by Andjic *et al*. (2007a). Sequence data for isolates collected from northern Australia were compared with those for *K. destructans* from Asia and the closely related species *K. eucalypti* and *K. viscidus* (Andjic *et al*. 2007b).

K. epicoccoides and three un-described *Kirramyces* spp. were found among isolates from Melville Island and Derby (Table 1). Nine isolates with an ITS2 profile similar to that of *K. destructans* were retained for further analysis. In both parsimony and Bayesian analyses, *K. eucalypti* was distant from *K. destructans* and *K. viscidus*, forming a clade with 100% bootstrap support (parsimony analysis) and a posterior probability of 1.0 (Bayesian) (Figure 3). Although related to *K. destructans*, isolates of *K. viscidus* resided in a clade discrete from those of *K. destructans* (Figure 3.).

Isolates of *K. destructans* from Asia grouped together, but were most closely related to those from Melville Island and the single isolate from Derby. There was only 1 bp difference in ßt and EF-1α sequences between the *K. destructans* isolates from Asia and those from Australia. ITS2 sequence data showed the greatest amount of variation with up to 4 bp difference between Asian isolates and some of the Australian isolates. Thus, from approximately 1000 bp of sequence, the maximum difference among isolates was 6 bp. This is within the normal limits of intra-species variation and justifies the identification of the Australian isolates as *K. destructans.*

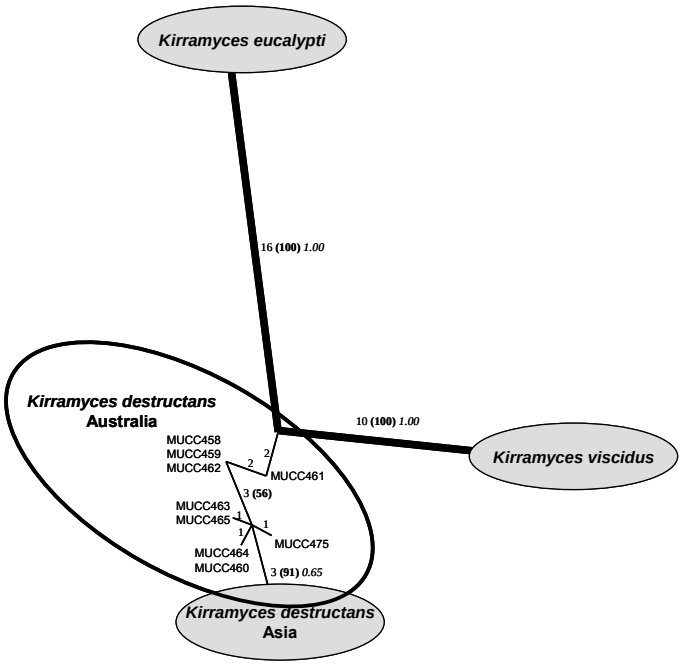


Figure 3. Unrooted phylogram of one of the 51 most parsimonious tree of 46 steps obtained from combined ITS, ßt and EF1-α sequence data. The numbers next to the branches represent bootstrap support (in brackets) and the posterior probabilities of the branch nodes based on Bayesian analysis. *Kirramyces eucalypti, K. viscidus* and *K. destructans* all represent strong supported terminal nodes.

The observed symptoms on leaves, conidial morphology, culture characteristics and multilocus sequence data lead us to conclude *K. destructans* is present in Australia. Variability amongst isolates from Melville Island suggests *K. destructans* is endemic to the region. This information has been provided to Biosecurity Australia and we believe it is appropriate to remove *K. destructans* from the Biosecurity Australia Watch List for eucalypt pathogens. Although, regular surveys have been conducted in northern Queensland and *K. viscidus, K. epicoccoides, K. eucalypti* and several, as yet,

undescribed *Kirramyces* spp. have been isolated (unpublished data), we have not irrefutably detected *K. destructans* in this region. Due to the potential impact of this pathogen on eucalypt plantations in tropical Australia, it is essential that monitoring in this region is maintained.

Table 1. *Kirramyces* species and isolates examined

Isolate	Name	Host	Origin	Collector	GenBank Accession no.		
					ITS	ß-tubulin	EF1α
CMW 22553	*Kirramyces destructans*	*Eucalyptus grandis*	Sumatra, Indonesia	PA Barber	DQ632667	DQ632625	DQ632732
CMW 17918	*K. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	DQ632666	DQ632624	DQ632731
CMW 19832	*K. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	DQ632665	DQ632623	DQ632730
CMW 17919	*K. destructans*	*E. urophylla*	Guangzhou, China	TI Burgess	DQ632701	DQ632622	DQ632729
MUCC 458	*K. destructans*	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU009634**	**EU009652**	**EU009643**
MUCC 459	*K. destructans*	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU009635**	**EU009653**	**EU009644**
MUCC 460	*K. destructans*	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU009630**	**EU009648**	**EU009639**
MUCC 461	*K. destructans*	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU009637**	**EU009655**	**EU009646**
MUCC 462	*K. destructans*	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU009636**	**EU009654**	**EU009645**
MUCC 463	*K. destructans*	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU009631**	**EU009649**	**EU009640**
MUCC 464	*K. destructans*	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU009633**	**EU009651**	**EU009642**
MUCC 465	*K. destructans*	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU009632**	**EU009650**	**EU009641**
MUCC 475	*K. destructans*	*Eucalyptus* sp.	Derby, Australia	MJ Wingfield	**EU009629**	**EU009647**	**EU009638**
MUCC 452, CBS 121156	*K. viscidus*	*E. grandis*	Mareeba, Australia	TI Burgess	EF031471	EF031483	EF031495
MUCC 453, CBS 121157	*K. viscidus*	*E. grandis*	Mareeba, Australia	TI Burgess	EF031472	EF031484	EF031496
MUCC 456, CBS 121155	*K. viscidus*	*E. grandis*	Mareeba, Australia	TI Burgess	EF031475	EF031487	EF031499
CMW 17917	*K. eucalypti*	*E. grandis x E. tereticornis*	New South Wales	AJ Carnegie	DQ632711	DQ632630	DQ632725
CMW 17916	*K. eucalypti*	*E. grandis x E. camaldulensis*	Queensland	AJ Carnegie	DQ632659	DQ632628	DQ632722
CMW 11687	*K. eucalypti*	*E. nitens*	New Zealand	M Dick	DQ240001	DS890168	DQ235115
MUCC 549	*K. epicoccoides*	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU117049**		
MUCC 550	*K. epicoccoides*	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU117050**		
MUCC 543	*Kirramyces* sp.	*E. grandis x E. urophylla*	Melville Island, Australia	TI Burgess	**EU009626**		
MUCC 544	*Kirramyces* sp.	*Eucalyptus* sp.	Derby, Australia	MJ Wingfield	**EU009628**		
MUCC 545	*Kirramyces* sp.	*Eucalyptus* sp.	Derby, Australia	MJ Wingfield	**EU009627**		

[1] Designation of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Cooperative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; MUCC = Murdoch University culture collection, Australia

APPENDIX III

New *Kirramyces* species

New *Kirramyces* species

Several studies over the past few years have revealed many new *Kirramyces* species in sub-tropical and tropical Australia that are yet to be described.

Table 1. New *Kirramyces* spp. used in phylogenetic study.

Culture no.[1]	Host	Location	Collector
MUCC 647	*E. dunnii*	Bundaberg, QLD	G. Whyte
MUCC 648	*E. dunnii*	Bundaberg, QLD	G. Whyte
MUCC 649	*E. dunnii*	Bundaberg, QLD	G. Whyte
MUCC 650	*E. dunnii*	Bundaberg, QLD	G. Whyte
MUCC 651	*E. dunnii*	Bundaberg, QLD	G. Whyte
MUCC 652	*E. dunnii*	Bundaberg, QLD	G. Whyte
MUCC 653	*Eucalyptus* sp	NSW	AC Carnegie
MUCC 654	*Eucalyptus* sp	NSW	AC Carnegie
MUCC 655	*E. urophylla*	Tiwi Islands, NT	TI Burgess
MUCC 656	*E. dunnii*	Bundaberg, QLD	G. Whyte
MUCC 657	*Eucalyptus* sp.	Derby, FNQ	TI Burgess
MUCC 658	*Angophora* sp.	Perth, WA	P Barber
MUCC 659	*Angophora* sp.	Perth, WA	P Barber
MUCC 660	*Eucalyptus* sp.	Derby, FNQ	TI Burgess
MUCC 661	*Corymbia* sp.	Perth,WA	GEStJ Hardy
MUCC 662	*Corymbia* sp.	Perth, WA	GEStJ Hardy
MUCC 663	*Corymbia. toreliana* x *C. variegata*	Kingaroy, QLD	AC Carnegie
MUCC 664	*Corymbia. toreliana* x *C. variegata*	Kingaroy, QLD	AC Carnegie
MUCC 665	*Eucalyptus* sp.	Mareeba, FNQ	TI Burgess
MUCC 666	*Eucalyptus* sp.	Mareeba, FNQ	TI Burgess
MUCC 667	*Corymbia. toreliana* x *C. variegata*	Kingaroy, QLD	AC Carnegie
MUCC 668	*E. dunnii*	Bundaberg, QLD	G. Whyte
MUCC 669	*E. dunnii*	Bundaberg, QLD	G. Whyte
MUCC 670	*Eucalyptus* sp.	Albany, WA	S Jackson
MUCC 671	*Eucalyptus* sp.	Albany, WA	S Jackson

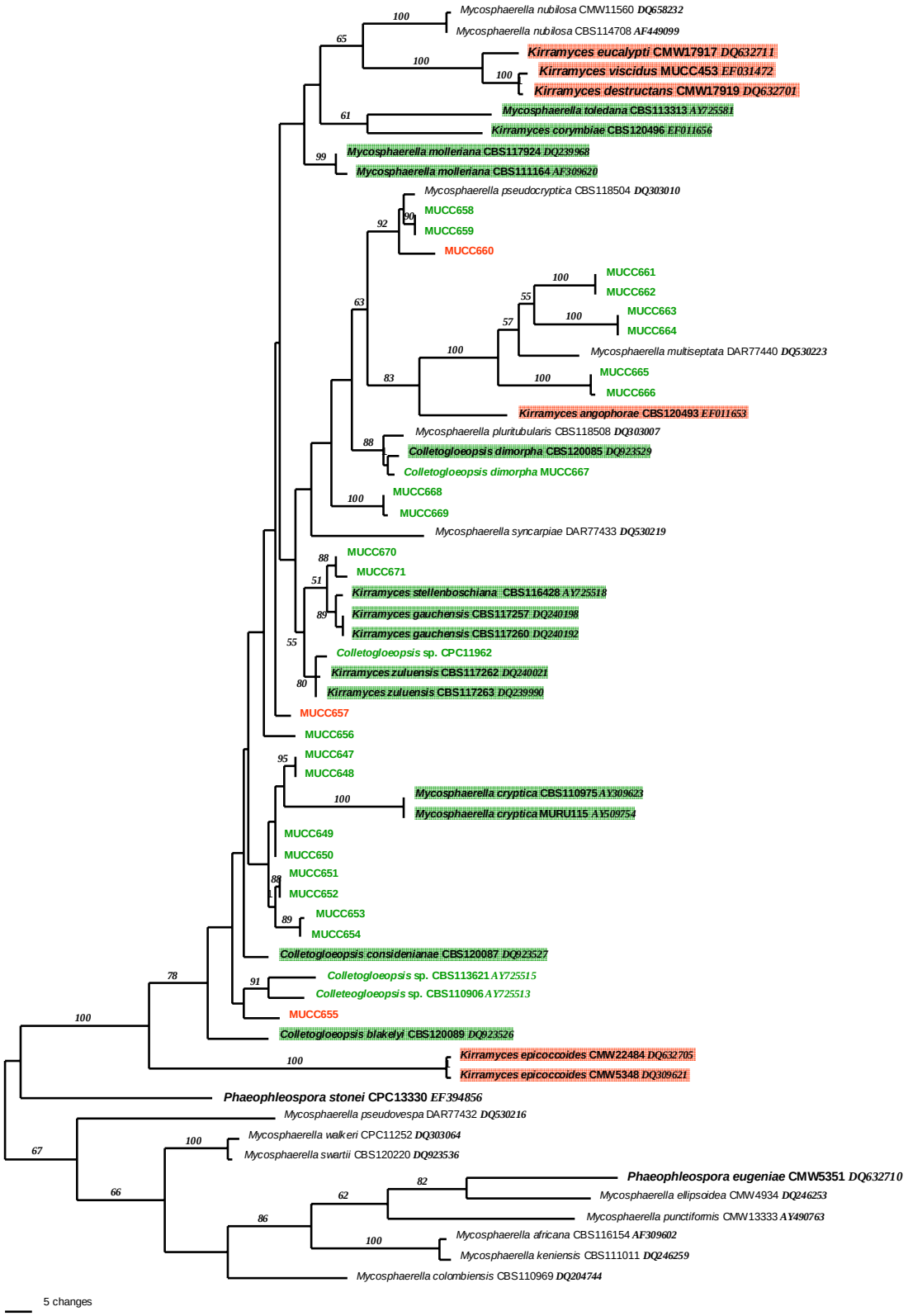


Figure 1. Known and yet un-described *Kirramyces* spp. occurring on eucalypts. One of 49 trees of 872 steps (CI=0.48, RI=077). Species with green background represent known *Colletogloeopsis*-like conidia type species; species in green represent undescribed *Colletogloeopsis*-like conidia type species; species with red background represent known species with *Phaeophleospora*-like conidia type species; species in red represent undescribed *Phaeophleospora*-like conidia type species. Several *Colletogloeopsis* species have been described since submission of the genus revision and these names have not yet been changed to *Kirramyces*.

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